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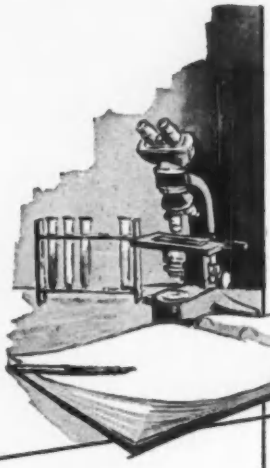

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CONTENTS

The effects of natural food-preservative substances on the development and survival of intestinal helminth eggs and larvae. I. Action on *Ascaris lumbricoides* eggs. CHIN THACK SOH. 1

The effects of natural food-preservative substances on the development and survival of intestinal helminth eggs and larvae. II. Action on *Ancylostoma duodenale* larvae. CHIN THACK SOH. 8

Isolation and study of a specific complement fixing antigen from adult *Schistosoma mansoni*. H. KENNETH SLEEMAN. 11

Naturally acquired yellow fever immunity in Muzo, Colombia. J. AUSTIN KERR, MANUEL ROCA-GARCIA AND JOHN C. BUGHER. 18

Intestinal nematodes in well-cared-for dogs. JOHN VAUGHN AND ROBERT JORDAN. 29

Phlebotomus (sandfly) fever viruses in tissue culture. J. R. HENDERSON AND R. M. TAYLOR. 32

The use of dithiazanine as a mass treatment for intestinal helminthiasis. LAUREANO GUERRERO, JORGE HOLGUIN AND DAVID BOTERO. 37

The prophylactic and curative activity of 1-maleinyl-4-(3′-chloro-4′-methylphenyl)-piperazine (Hoechst S 688) in experimental schistosome infections. GEORGE W. LUTTERMOSER, JOHN I. BRUCE AND DONALD B. MCMULLEN. 39

An enriched, egg extract medium for recovering viable *Entamoeba histolytica* trophozoites from fecal suspensions kept at room temperature for four days. NEVA N. GLEASON, MORRIS GOLDMAN AND R. K. CARVER. 46

In vitro studies on the sites of Japanese encephalitis virus multiplication in the heron, an important natural host in Japan. WILLIAM F. SCHERER AND R. P. J. SMITH. 50

A comparative study of the subcutaneous inoculation of germfree and conventional guinea pigs with two strains of *Trichomonas vaginalis*. WALTER L. NEWTON, LUCY V. REARDON, AND ANNA M. DELEVA. 56

Isolation of Germiston virus, a hitherto unknown agent, from culicine mosquitoes, and a report of infection in two laboratory workers. R. H. KOKERNOT, K. C. SMITHBURN, H. E. PATERSON, AND B. M. MCINTOSH. 62

Kairi virus, a new virus from Trinidadian forest mosquitoes. C. R. ANDERSON, T. H. G. AITKEN, L. P. SPENCE, AND W. G. DOWNS. 70

A new antigenic group of arthropod-borne viruses. The Bunyamwera group. JORDI CASALS AND LORING WHITMAN. 73

Manzanilla virus: A new virus isolated from the blood of a howler monkey in Trinidad, W. I. C. R. ANDERSON, L. P. SPENCE, W. G. DOWNS, AND T. H. G. AITKEN. 78

Electrophoretic pattern of hyperproteinemic sera in a population group of rural Panama. G. ARROYAVE, N. S. SCRIMSHAW, O. PINEDA AND M. GUZMÁN. 81

Ecological studies of wild rodent plague in the San Francisco Bay area of California. III. The natural infection rates with *Pasteurella pestis* in five flea species during an epizootic. S. F. QUAN, V. I. MILES, AND L. KARTMAN. 85

Ecological studies of wild rodent plague in the San Francisco Bay area of California. IV. The fluctuation and intensity of natural infection with *Pasteurella pestis* in fleas during an epizootic. S. F. QUAN, L. KARTMAN, F. M. PRINCE, AND V. I. MILES. 91

Ecological studies of wild rodent plague in the San Francisco Bay area of California. V. The distribution of naturally infected fleas during an epizootic in relation to their infection rates. L. KARTMAN, S. F. QUAN, AND V. I. MILES. 96

Book reviews: *Preventive Medicine: Principles of Prevention in the Occurrence and Progression of Disease*, by Hilleboe and Larimore, ed'rs; *Microbiology—Yesterday and Today. A Symposium*, by Bryson, ed'r; *Malariology, with Special Reference to Malaya*, by Sandosham; *A Synopsis of Skin Diseases*, by Solomons, Jr.; *Principles of Administration Applied to Nursing*, by Goddard. 101

Books received. 103

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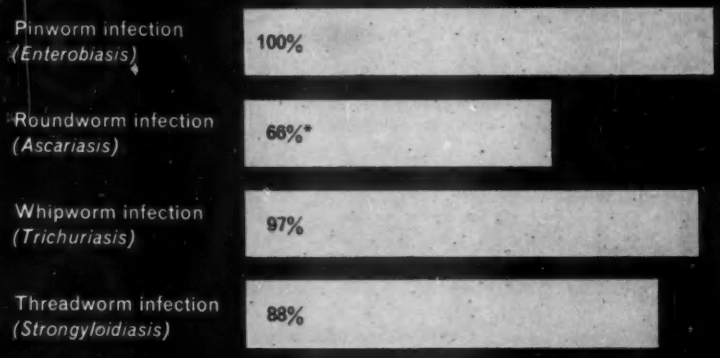

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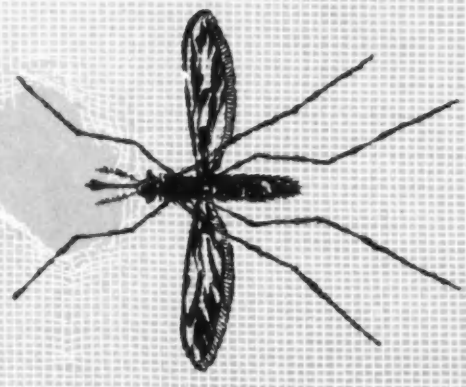

THE EFFECTS OF NATURAL FOOD-PRESERVATIVE SUBSTANCES ON THE DEVELOPMENT AND SURVIVAL OF INTESTINAL HELMINTH EGGS AND LARVAE

I. Action on *Ascaris lumbricoides* Eggs*

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In areas where nightsoil is commonly used, helminth eggs and larvae may be readily demonstrated on fresh vegetables at the market.[1] It is therefore reasonable to assume that in those areas the prevalence of intestinal helminths is in part due to the ingestion of infective stages adherent to uncooked food-plants. Where refrigeration and commercial canning are little used, vegetables prepared or preserved in chemical substances constitute a considerable proportion of the total food consumed. In such foods, the development and survival of the infective stages of helminths exert an undetermined influence on the prevalence of these parasites.

Reported observations indicate that none of the common preservatives such as salt, sugar, vinegar, alcohol and bean sauce in the usual concentrations can be relied upon to kill the eggs of *Ascaris lumbricoides*. Combinations of these preservatives, with or without the addition of spices and other substances as actually used by the people, have generally been found to be well tolerated by the larvae and early developmental stages.[1] The present experiments were designed to include tests of a greater number of substances, under a wider range of conditions than have been used in previous studies, in an attempt to obtain such information as possibly would lead to practical improvements in food preservatives, or would provide a basis for choosing preserved foods for consumption in situations where contamination from soil and from handling must be regarded as probable.

MATERIALS AND METHODS

Fertile eggs were concentrated from fresh stools by repeated centrifugal sedimentation in distilled water. No chemicals were added. Most of the coarser debris was removed by straining through four-ply gauze before the second washing. Eggs collected in this manner were incubated in open glass bottles in clean water about 2 cm in depth, stored in a dark cabinet at room temperature. Every 2 days the water was changed to prevent putrefaction and fermentation, and under these conditions development reached the infective larval stage with its clearly discernible molted cuticle in about 3 weeks.

Freshly collected eggs and those containing infective larvae were tested in the following substances in aqueous solutions or infusions, the powdered and ground materials being packaged in porous bags which allowed the soluble elements to diffuse into the medium, and the percentage dilutions being computed by volume for the liquids and by weight for the solids: (a) *salt* (NaCl—1%, 10%, 30%; (b) *sugar* (white granulated sucrose)—0.1%, 1%, 5%, 10%, 30%, 50, 80%; (c) *vinegar* (Heinz apple cider vinegar containing 5% acetic acid)—50%, 100%; (d) *acetic acid*—5%, 10%; (e) *ethyl alcohol*—5%, 10%, 30%, 40%; (f) *bean sauce* (Chinese variety) —50%; (g) *onion* (fresh mature bulb cut into 1–3 mm pieces)—1%, 5%, 20%; (h) *garlic* (same as onion)— 1%, 5%, 20%; (i) *mustard* (powdered)—1%, 5%, 10%; (j) *red pepper* (powdered) —1%, 5%, 10%; (k) *cloves* (B. Fisher & Co., ground)—1%, 5%; (l) *allspice* (B. Fisher & Co., ground)—1%, 5%, (m) *cinnamon* (McCormick Co., ground)—1%, 5%; (n) *ginger* (crushed)— 1%, 10%.

The survival of unhatched infective larvae and the development of freshly collected eggs were also tested in certain combinations of the above substances as they are commonly used for vegetable pickling according to Oriental and Western recipes, and in a number of modifications of these formulas. The standard formulas were:

(1) Oriental recipe—100 gm chopped cabbage,

* From the Department of Tropical Medicine and Public Health, Tulane Medical School, New Orleans; supported in part by Research Grant B-752 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.

salted 12–13%, by weight; 1 gm each of red pepper, onion, garlic and ginger; 100 ml water.

(2) Western recipe—100 gm chopped cabbage, salted 10% by weight; 30 gm granulated sugar; 1 gm each of cinnamon, cloves, mustard and shredded horse-radish; 0.4 gm allspice; water 70 ml; vinegar, 3 ml added to the surface after the above is mixed; seal in glass container.

For tests of individual substances, 50 ml groundglass-stoppered bottles were used. After the bottles were nearly filled, 50,000 to 100,000 eggs in packed sediment from centrifuged suspensions were added, stoppers were inserted firmly, the suspensions mixed by gentle shaking, and stored in a dark cabinet at 22-28°C. Controls in water were similarly prepared.

Samples from each test-suspension were examined at 10, 20, 30 and 40 days. After gentle shaking, a 2–3 ml portion of the suspension was transferred to a centrifuge tube. After washing once, a portion of the sedimented eggs was transferred to a slide for microscopic examination. The fresh and infective eggs were handled differently. For examination of the former, a drop of 0.5% aqueous solution of sodium hypochlorite was added to the egg suspension and mixed before placing the coverglass. This removed the outer shell membranes making it possible to readily determine the degree of development, if any. If no development had taken place, the remainder of the washed sample was placed in clean water and re-examined after 15–20 days. For examination of infective eggs, sodium hypochlorite usually was not necessary. When on the basis of motility there was any doubt about viability, the remainder of the washed sample (1,000 eggs or more) was inoculated into a mouse which was then examined for larvae and lesions in the liver and lungs, 2–4 days later. Control inoculations were made using eggs from water cultures of equal age.

RESULTS

Except where otherwise stated, the terms "larvae" and "eggs" refer respectively to infective second-stage larvae inside the egg shell and to the ova or developmntal stages in the egg shell.

In the controls, essentially all of the larvae at 40 days were living and capable of infecting mice; the fresh eggs developed normally to the infective larval stage.

Among various substances tested, garlic and mustard were the most effective in killing the larvae and in preventing development of the egg. Even in low concentrations both stages were killed in 30 days or less; in higher concentrations most of the larvae and eggs were dead at 10 days (Table 1). Several other substances apparently were toxic to both stages. The larvae had survived poorly or not at all at 40 days in the higher concentrations of onion, bean sauce, clove, allspice and cinnamon. Development was retarded or entirely prevented so that the infective stage was not reached at 40 days in any except acetic acid, salt, sugar, and low concentrations of alcohol, clove and allspice. In red pepper the larvae survived as well as in the controls but the egg showed little or no development (Table 1).

In neither of the pickling mixtures, nor in any modification of them, was there good survival of the larvae or development of the egg beyond the cleavage stages (Table 2). In the Oriental mixture, which contained garlic, onion, red pepper, ginger and salt, most of the infective stages were dead at 30 days and none survived for 40 days. In all other mixtures or modifications there was poor survival, but in each instance a few infective larvae could be demonstrated by inoculation into mice. The addition and withdrawal of various ingredients made relatively slight differences in the results except in the case of garlic and mustard, and even with these the results were somewhat inconsistent. At 15 days some of the eggs in Oriental mixture were still capable of developing to the infective stage when transferred to clean water but at longer periods they were not, and in all other mixtures tested the eggs were so damaged that little or no further development occurred when they were removed to clean water. It is to be noted that all of the mixtures tested contained 1% or more of at least one of the substances found to be most toxic in the first trials, i.e., garlic, mustard or onion (Tables 1 and 2).

Further testing of garlic and mustard in 1% aqueous infusions, with and without the addition of acetic acid and under various temperature conditions, gave results that appear to be somewhat inconsistent with the previous tests (Table 3). In plain aqueous infusions (1 gm/100 ml water) of both garlic and mustard there was no

TABLE 1

Survival of the infective stage and development of the egg of Ascaris lumbricoides *in various food-preservative substances*

Material	%	Larva survival* at days				Egg development† at days			
		10	20	30	40	10	20	30	40
Salt	1, 10	+++	+++	+++	+++	++	+++	+++	+++
	30	++	++	++	++	+	++	+++	+++
Sugar	1	+++	+++	+++	+++	++	+++	+++	+++
	5	+++	+++	+++	+++	++	++	++	++
	10	+++	+++	+++	+++	++	++	++	+++
	30–80	+++	+++	+++	+++	++	+++	+++	+++
Vinegar	50–100	+++	+++	+++	+++	++	+++	+++	+++
Acetic acid	5, 10	+++	+++	+++	+++	++	+++	+++	+++
Ethyl alcohol	5, 10	+++	+++	+++	+++	+	+++	+++	+++
	30, 40	++	++	++	++	+−	++	(++)	(++)
Bean sauce	50	++	++	+	+−	+−	+−	+−	+−
Onion	1	+++	+++	++	++	+	++	++	++
	5	+++	++	++	++	+−	+−	+−	+−
	20	++	+	+	−	+−	−	−	−
Garlic	1	++	+	−	−	+−	−	−	−
	5	++	+−	−	−	+−	−	−	−
	20	+	+−	−	−	+−	−	−	−
Mustard	1	++	++	++	+	+	++	(++)	(++)
	5	++	+	−	−	(+)			
	10	+	+−	−	−	(+)	(+)	(+)	(+)
Red pepper	1, 5	+++	+++	+++	+++	+	+	++	++
	20	+++	+++	+++	+++	+−	+−	+−	+−
Cloves	1	++	++	++	+−	+	+++	+++	+++
	5	++	++	++	+−	+	(++)	(++)	(++)
Allspice	1	++	++	+	+	+	+++	+++	+++
	5	++	++	+	+−	+	++	++	(++)
Cinnamon	1	++	++	+	+	+	++	++	++
	5	++	++	+−	+−	+	++	++	++
Ginger	1	+++	+++	+++	++	+	+	+	+
	10	++	++	++	++	+	+	+	+

* Survival of infective-stage larvae in the egg: +++ essentially all living; ++ considerable mortality but half or more living; + heavy mortality, less than half living; +− mostly dead, few viable by mouse test; − none viable.

† Development of eggs: +++ complete to infective larva; ++ advanced cleavage; + early cleavage; +− no development but viable; − not viable; ( ) did not become infective when transferred to water.

TABLE 2

Survival of the infective stage and development of the egg of Ascaris lumbricoides *in pickling mixtures, with chopped cabbage added*

Pickling mixture*	Larva survival† at days			Egg development† at days		
	15	30	40	15	30	40
Oriental, unmodified	++	+−	−	++	(++)	(++)
− garlic	+++	++	+−	(+)	(+)	(+)
− onion	++	+−	+−	(+)	(+)	(+)
− red pepper	++	+−	+	(+)	(+)	(+)
− ginger	+	+−	+−	(+)	(+)	(+)
+ mustard	++	+−	+−	−	−	−
+ cinnamon	+−	+−	+−	(+)	(+)	(+)
+ cloves	+	+−	+−	(++)	(++)	(++)
+ allspice	+	+−	+−	(++)	(++)	(++)
+ 3 above	+−	+−	+−	(++)	(++)	(++)
Western, unmodified	+−	+−	+−	(+)	(+)	(+)
− mustard	+−	+−	+−	(++)	(++)	(++)
+ garlic	+−	+−	+−	−	−	−
− cinnamon	+	+	+	(++)	(++)	(++)
− cloves	++	++	+	(++)	(++)	(++)
− allspice	+	+	+	(++)	(++)	(++)
− 3 above	++	+	+	(++)	(++)	(++)

* See Materials and Methods for formula. Amounts added were 1% by weight.
† See Table 1 for scoring of survival and development.

TABLE 3

Survival of larvae and development of eggs of Ascaris lumbricoides *in 1 per cent infusions of garlic and mustard under various conditions*

Test conditions	Larva survival* at days				Egg development* at days			
	10	20	30	40	10	20	30	40
Garlic								
Tested at 22–28°C	+++	+++	++	++	−	−	−	−
Tested at 4–6°C	+++	+++	+++	+++	+−	+−	+−	+−
After 30 minutes boiling, tested at 22–28°C	+++	+++	+++	+++	+−	+−	++	+++
1% acetic acid added, tested at 22–28°C	+++	+++	+++	+++	++	+++	+++	+++
Mustard								
Tested at 22–28°C	+++	+++	+	+	−	−	−	−
Tested at 4–6°C	+++	+++	+++	+++	+−	+−	+−	+−
After 30 minutes boiling, tested at 22–28°C	+++	+++	++	++	−	−	−	−
1% acetic acid added, tested at 22–28°C	+++	+++	+++	+++	++	++	+++	+++

* See Table 1 for scoring of survival and development.

apparent effect on the larvae at 20 days, and at 30 and 40 days survival was greater than previously obtained, especially with the garlic. Lowering the temperature, boiling the infusions before use, and adding 1% acetic acid each failed to have any marked effect on the results with the larvae. However, when applied to eggs freshly isolated from feces, the infusions of both substances were as effective as formerly in killing the eggs even within 10 days at room temperature and they were nearly as effective at low temperatures. Most noteworthy were the different results when the infusions were boiled for 30 minutes before use and when acetic acid was added. The effectiveness of garlic was less after boiling but that of mustard was undiminished. When acetic acid was added, both garlic and mustard were rendered completely ineffective.

DISCUSSION

Depending upon the nature of the soil and the type of rainfall, the eggs of *A. lumbricoides* are destroyed rather rapidly in cultivated plots.[2] However, some of the eggs may persist throughout the growing season, and due to the splashing action of rain drops, eggs are likely to occur on both rooty and leafy vegetables grown on contaminated soil. Attachment of viable eggs onto vertical surfaces up to 30 centimeters above the soil surface has been observed.[3]

Since vegetables from contaminated soils bear eggs in different stages of development, an ideal pickling mixture or preservative must be lethal to infective-stage eggs and must prevent the earlier stages from becoming infective. The latter would be unimportant if killing of the infective stage were relatively prompt and certain. While information gained from previous reports and from the present investigation does not suggest a completely reliable ovicidal food-preservative, certain of the findings are promising.

Previous studies on eggs of *A. lumbricoides* in salt solutions have indicated that development proceeds at all tested concentrations up to saturation,[4, 5, 6] but when the infective stage is reached the larvae soon die.[7] In the present study observations were not carried beyond 40 days. Up to that time there was very little mortality of larvae in salt solutions close to saturation (30%), and the developing eggs reached infectivity at 30 days. It is of interest that in the heavy brine eggs floated at the surface only temporarily. As they settled, some became slightly collapsed but many seemed to be undamaged. The same phenomenon was observed in the tests of sugar.

Sugar (sucrose) solutions were found by Yagi[6] and in the present study to be well tolerated by *Ascaris* eggs. He found that in 1–10% solutions the eggs remained viable but did not develop until transferred to water. This was also observed in the present study in the 5% solution but in the 10% solution development proceeded slowly, reaching the infective stage only after 40 days of incubation. It is noteworthy that in both experiments 5% sugar solution barred development beyond the cleavage stages whereas the 1% and 30% solutions did not.

Vinegar and equivalent aqueous solutions of acetic acid, both of which quickly kill the cysts of *Entamoeba histolytica* and other enteric microorganisms,[8] have little if any damaging effect on the eggs and unhatched larvae of *Ascaris*. Yagi[6] reported that in full strength vinegar eggs developed to the morula stage and died, but in a half-strength dilution the infective stage was reached in 25 days. Asada[9] observed no effect of undiluted vinegar on the infective stage in 7 days. Using approximately equivalent concentrations of acetic acid, Ohba[10] observed that all larvae died in 30 days, but Yoshida[5] and Jettmar and Exner[11] obtained survival and development to the larval stage in solutions of 10–12.5%. This is in agreement with present results which showed rapid full development of the eggs within 20 days and good survival of the larvae for 40 days in full strength vinegar and in 10% acetic acid.

Ethyl alcohol in various concentrations has been tested by several workers. Yoshida[5] and Jettmar and Exner[11] found that unembryonated eggs of *Ascaris* survived at least one week in 50% alcohol, and Yagi[6] found that development to the infective stage occurred in concentrations up to 20% but not in stronger solutions. Findings in the present study are in general agreement with those of others. Development occurred in the weaker solutions but in 30 and 40% solutions there was only slight development and the eggs were all dead at 30 days. Larvae were undamaged at 40 days or less in the weak solutions and even in 40% solutions there was 50–75% survival. It is thus apparent that unless its action is indirect in the presence of other substances, alcohol in the

usual concentrations would have relatively little value as an ovicide in food preservatives.

Oriental bean sauce at half-strength did not permit development of eggs and was a moderately unfavorable medium for larval survival. Possibly due to variations in the preparations used, these results do not agree with those of Yagi[6] who obtained fully developed larvae at 28 days in the half-strength sauce. Asada[9] reported infectivity of larvae after immersion for 7 days in full-strength sauce.

Among the most active substances tested in the present investigation, mustard is the only one on which comparable studies have been reported; in this case there is close agreement in the results. Yagi[6] observed no development in infusions of 5–20%, and he showed by recultivation in water that exposure for 23 days killed the unembryonated eggs. He did not test the survival of infective larvae. Kunzii[12] recently has shown that each of five different kinds of mustard oil, in extremely low concentrations ($1:10^{-4}$), decreases oxygen consumption of the developing egg in direct proportion with its ovicidal effect. Yagi[6] reported observations on the ovicidal properties of one other of the present series of test substances—ginger—but owing to uncertainty about the concentration of the stock infusions, direct comparison of results can not be made. As was also true of clove, allspice and cinnamon infusions, the soluble or diffusible substances in ginger, if sufficiently concentrated, interfered with development. The spices also appeared to be toxic to the infective larvae although there was in all tests some survival up to 40 days.

Precise results on which firm conclusions can be drawn were not obtained from tests of any of the substances which appeared to be the most active. When tested alone or in combination with other substances in pickling mixtures it was apparent that garlic, onion and mustard contained water-soluble or diffusible elements that either killed or retarded the development of *Ascaris* larvae. The quantities of these substances and the conditions necessary for reliance on the absence of infective-stage eggs within a given period have not been satisfactorily determined. Temperature of the medium and stage of development of the eggs are well recognized important variables which influence the results. The most noteworthy factors not previously determined are those introduced by combining with the toxic substances other chemicals which interfere with their action. It was clearly demonstrated that acetic acid, which is itself highly active in killing certain bacteria and amebic cysts, in some manner is protective of larvae or developmental stages within the eggs of *Ascaris* when exposed to the action of garlic and mustard. Whether the acetic acid alters these substances directly or renders the egg's shell impermeable to them is not known.

Variation in shell permeability may have been a factor in the unexpected negative results when garlic and mustard were tested under various conditions against infective eggs (Table 3). In this instance the infusions were as active against the developmental stages as in previous tests, but were markedly less active against eggs containing infective larvae. It is possible that there was some degree of failure in attempts to maintain uniform conditions in the washing, culturing and testing procedures, but it is also possible that variable conditions in the bowel and in the feces may render the shells of different collections of eggs more or less permeable to individual substances.

Contrary to initial expectation, the pickling mixtures of several substances, each more or less damaging to the larval and developmental stages, did not prove appreciably more effective than the individual components. One possible exception was the addition of mustard to the Oriental pickling mixture which contained garlic, and the addition of garlic to the Western mixture which contained mustard (Table 2). Otherwise the results were essentially the same for all combinations containing either garlic or mustard. In a medium containing several complex substances, including the products of bacterial fermentation, it is difficult to identify all of the important factors. The pH of the Oriental cabbage pickle was 4.2 at 40 days. According to Otake[13] the optimum for development of *Ascaris* eggs is pH 5.0–7.0, and Schmelewa[14] observed no retardation of development within the range of pH 3 to pH 8. Takano[15] reported that acid environments are more favorable than alkaline ones but this can not be accepted without qualification since incubation of *Ascaris* eggs in relatively strong solutions of sodium hydroxide is a highly successful routine procedure.

Since the developing *Ascaris* larva demands oxygen in good supply up to the completion of its molt within the egg, but thenceforth requires very little oxygen for its survival, this circum-

stance must be taken into account in interpreting results, especially when the oxygen supply has been limited in closed containers or by bacterial fermentation.[16] It was with this in mind that recultivation of the treated eggs was included in the experimental procedures. In only those trials in which treated eggs did not become infective when transferred to water can the tested substance be regarded as having permeated the egg and killed the ovum, embryo or immature larva. It is on this basis that the toxicity of garlic, mustard and onion, and to a lesser degree clove and allspice, appeared to be demonstrated.

SUMMARY

Various substances commonly used as food preservatives were tested for ability to kill or prevent the development of infective larvae of *Ascaris lumbricoides*. Eggs for the tests were washed without chemicals from human stools and the infective-stage eggs were obtained by culturing in water for 3 weeks or longer.

In aqueous solutions at concentrations adaptable to practical situations, salt, sugar, vinegar (acetic acid) and ethyl alcohol were essentially without effect on either the infective or developmental stages. At 40 days in half-strength bean sauce there was relatively poor survival of larvae and no development beyond the one-cell stage, although eggs remained viable. In 1% garlic, 5% mustard and 20% onion infusions, infective larvae were killed in 40 days or less; eggs were killed in 20 days or less. Infusions of up to 20% red pepper had no effect on the infective stage and did not kill the eggs, but did bar their development. In 5% infusions of clove, allspice, and cinnamon and 10% ginger for 40 days, there was 50% or less survival of the infective larvae, and eggs did not develop beyond the cleavage stages; some of the eggs were still viable in the cinnamon and ginger, however, as demonstrated by recultivation in water.

Tests on mixtures of the various substances according to pickling recipes, on certain modifications of these formulas, and on garlic and mustard infusions under various conditions indicated again that garlic and mustard are toxic to both larval and developmental stages, that there is little if any enhancement of the killing effect by combining these with less toxic substances, that vinegar and 10% acetic acid interfere with the killing action of garlic and mustard, and that different collections of eggs appear to vary in their susceptibility to the action of garlic and mustard.

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THE EFFECTS OF NATURAL FOOD-PRESERVATIVE SUBSTANCES ON THE DEVELOPMENT AND SURVIVAL OF INTESTINAL HELMINTH EGGS AND LARVAE*

II. Action on *Ancylostoma duodenale* larvae

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Experimental studies have shown that *Ancylostoma duodenale* infections frequently may be acquired by ingestion of infective larvae.[1] Recent reports suggest that infections with this species may be more readily established by oral than by cutaneous inoculations, whereas with *Necator americanus* the reverse is true.[2, 3] In areas where night soil is commonly used, viable eggs and larvae of the hookworms and other intestinal helminths can be recovered from marketed fresh vegetables.[4, 5, 6] In Korea and elsewhere, owing in part to the lack of other means of preservation, a significant proportion of these fresh vegetable crops are pickled, fermented in brine, or stored for short periods in sauces and solutions or infusions containing various spices and other substances which prevent spoilage.

The possibility of development and survival of hookworm larvae in food-preservative substances has been studied briefly by Hahn and Koh[7] who found that a pickling mixture containing garlic, red pepper and onions promptly killed the infective-stage larvae. In the present study, the question of development and survival of hookworm larvae in preserved foods has been examined more extensively, including in the tests all of the more commonly used natural food-preservative substances.

MATERIALS AND METHODS

Eggs were obtained in fresh stools from an individual known to harbor only *Ancylostoma duodenale*. After repeated straining and centrifugal sedimentation in water, the remaining fecal debris was just adequate to promote larval growth in aqueous cultures. No chemicals were used in the washing-concentration procedures. Advanced first- and early second-stage rhabditoid larvae were obtained from shallow water cultures after three days at 24–28°C. Third-stage filariform (infective) larvae were cultured in damp granular charcoal and were collected by the damp-pad method.[8] Nearly all of the larvae collected in this manner retained the sheath.

The freshly collected eggs and larvae were immersed for various periods in aqueous solutions or infusions of the following substances, the powdered materials being packaged in gauze bags to facilitate recovery and examination of eggs and larvae, and the percentage dilutions (shown in Table 1) being computed by volume for the liquids, by weight for the solids: *Salt* (sodium chloride), *sugar* (white granulated sucrose), *acetic acid*, *ethyl alcohol*, Chinese soya *bean sauce*, *onion* (1–3 mm cubes), *garlic* (1–3 mm cubes), powdered yellow *mustard*, powdered *red pepper*, ground *cloves*, ground *allspice*, ground *cinnamon*, and crushed *ginger*.

In addition to the individual substances, tests were run on mixtures of them combined according to Oriental and Western pickling recipes, and on variations of these recipes as follows: (1) Oriental pickle—100 gm chopped cabbage salted 1:8; 1 gm each of red pepper, onion, garlic, and ginger; 100 ml water. (2) Western pickle—100 gm chopped cabbage salted 1:10; 30 gm sugar; 1 gm each of mustard, cloves, allspice and ginger; 70 ml water; and 3 ml vinegar poured over the surface after the above are mixed. (3) Oriental and Western pickling mixtures modified by adding (if lacking) or omitting the following ingredients: onion, garlic, mustard, red pepper, clove, allspice, cinnamon, and ginger, each separately and the last 3 together (from Western mixture).

The tests of individual preservatives and of

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pickling mixtures were run in 50 and 200 ml wide-mouth glass jars respectively, the solid materials being well mixed and allowed to throughly diffuse (at least 3 hours) before testing. All tests were run at 24–28°C. A few drops of heavy suspensions of the eggs or larvae were added to the test fluids, the bottles were then stoppered, and the suspensions stirred by rotation. After specified periods of exposure, samples were pipetted from the bottom, washed in water by dilution and centrifugation, transferred to a slide and examined microscopically. To test viability, developing eggs were reincubated in shallow water. Larvae having normal appearance but motionless were regarded as dead if they failed to respond to heat stimulation after additional washing.

RESULTS

In all concentrations of practical value, none of the more common food-preservative materials permitted full development of either the eggs or rhabditoid larvae. There was complete development of larvae in the eggs tested in 1% infusions of onion, allspice and ginger but even in these the rhabditoid larvae failed to survive (Table 1). It is thus evident that preinfective stages of *A. duodenale* in foods preserved in these materials would not be infective when the foods were eaten. Pickling mixtures and all of the various tested modifications of them also were too toxic for developmet of larvae.

Infective larvae were much more resistant than the developmental stages (Table 1). In half-strength bean sauce and 1% infusions of the spices there was survival for 2 days or longer. Only garlic and mustard or pickling mixtures containing one of these substances proved to be highly toxic to the infective larvae. Survival was especially limited in infusions containing mustard. Whereas in 1% mustard infusions, or in Western pickling mixtures containing mustard, survival was limited to only a few hours, larvae lived for two days in mixtures containing all ingredients except mustard. Infusions containing 20% garlic were nearly as toxic as 1% mustard.

Infusions of 1% mustard were rendered less toxic by boiling for 30 minutes but were unaltered by the addition of 1% acetic acid. In the infusions of mustard alone or with acetic acid added the larvae survived only 12 hours, while in the boiled infusion some of them were still motile after 3 days of exposure. Similarly, in 1% garlic infusions the larvae lived for only 1 or 2 days but survived

TABLE 1

Survival of eggs and larvae of Ancylostoma duodenale *in food-preservative substances*

Materials	%	Maximum survival in days		
		Eggs	1st stage larvae*	Infective larvae
Salt	10	3	1	5
Sugar	30	3	1	2
Acetic acid	1	5	1	5
Ethyl alcohol	10	3	1	3
Bean sauce	50	5	1	2
Onion	1	3	1.5	3
Onion	20	3	1.0	2
Garlic	1	3	1.0	2
Garlic	20	3	1	1
Mustard	1	3	1	1
Red pepper	1	10 (prelarva)	1.5	5
Red pepper	20	10 (prelarva)	1.0	5
Cloves	1	3	1	2
Allspice	1	hatched at 10	1	2
Cinnamon	1	3	1	2
Ginger	1	hatched at 3	1.5	10
Ginger	20	5	1.0	3
Water controls	–	hatched at 3	infective at 5	10

* Examined at 12-hour intervals.

up to 5 days in infusions that had been boiled for 30 minutes.

DISCUSSION

The results indicate that only the infective stage of *A. duodenale* can survive in the common food preservatives long enough to play any part in the transmission of infections. The eggs developed and hatched in low concentrations of onion, allspice, and ginger, but neither in these nor in any other substance or mixture was there any development of the early larval stages.

The most toxic of the tested substances were mustard and garlic. It was found that the toxicity of these two substances was very appreciably diminished by boiling for 30 minutes but not by the addition of acetic acid as was earlier found in tests on *Ascaris lumbricoides*.[9] Moreover, it was found that pickling mixtures containing 1% or more of either garlic or mustard in addition to any combination of the other common ingredients of pickling or preservative mixtures killed the infective-stage larvae within a few hours, or at most 1 or 2 days. Using the pure oil of mustard, Yasuda[10] found that all larvae of *A. duodenale* and other hookworms were killed in 24 hours or less in dilutions up to 1 in 32,000.

In view of these observations and those described elsewhere[9] it appears that whereas the infective stages of *A. lumbricoides* may persist in preserved foods and play at least a minor role in the transmission of infection, hookworm larvae do not. If mustard or garlic or both are among the ingredients and acetic acid is not, foods preserved in pickling mixtures for periods of several weeks generally will contain no infective larvae of hookworms and very few if any of *A. lumbricoides*.

SUMMARY

Eggs, rhabditoid larvae and infective-stage larvae of *Ancylostoma duodenale* were immersed in common food-preservative substances individually and in combinations used in pickling mixtures. In all tests, the eggs and rhabditoid larvae failed to develop to infective-stage larvae. Infusions containing the usual amounts or less of mustard or garlic used in food preservatives were especially toxic, and combinations of either of these substances with other commonly used preservatives such as salt, sugar, acetic acid, bean sauce and spices were equally or more toxic to all stages, killing the infective-stage larvae within a few hours or at most 1 to 2 days.

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ISOLATION AND STUDY OF A SPECIFIC COMPLEMENT FIXING ANTIGEN FROM ADULT *SCHISTOSOMA MANSONI*

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The usefulness of the complement fixation test in the diagnosis of schistosomiasis has been limited by the frequence of non-specific reactions. While the apparent solution of this problem lies in the purification of the antigenic components, relatively few investigations have been conducted along this line. Attempts to isolate antigens from adult *Schistosoma mansoni* have consisted primarily of extractions with organic solvents, buffered saline solutions, or occasionally, a combination of the two. Davies and Eliakim[1] investigated the extracting potential of a number of organic solvents and Coca's solution, and found the latter to be the superior extractant.[2, 3] Chaffee et al.[4] effected some reduction of components responsible for cross reactions with syphilitic antisera by treating the adult worms with anhydrous ether prior to extracting with buffered saline. Sodium desoxycholate solution was used by Schneider et al.[5] to prepare a complement fixing antigen, and later the serologically active component was precipitated by 80% ethanol.[6] Despite the improvements achieved in these antigens, their usefulness was limited by persistent cross reacting components, and in the latter case, low antigenic activity.

Preliminary investigations resulted in the selection of sodium desoxycholate as the initial extractant. Chemical fractionation of the sodium desoxycholate soluble components permitted the isolation of an antigen specific for schistosomiasis. The serologic properties of the purified antigen were evaluated in a quantitatively standardized complement fixation procedure, and the structural components studied by physiochemical methods.

MATERIALS

S. mansoni: Adult worms were recovered by an 0.85% sodium chloride perfusion of the mesenteric-portal system of experimentally infected hamsters. The worms were washed three times with 0.85% saline, three times with distilled water, lyophilized, and stored under vacuum at −40°C.

Extracting Solution: The sodium desoxycholate solution (Na DOC) was a modification of that described by McCarty and Avery.[7] It contained 0.1 M sodium chloride, 0.2 M sodium citrate, and 0.6 % sodium desoxycholate. The 0.1% Na DOC concentration employed by McCarty and Avery was inadequate for the extraction of antigen from schistosomes. The two extracting solutions for the purification of the antigen were those used in Method 10 of Cohn et al.[8] for the extraction of gamma globulin, and fraction III-O respectively. Solution I contained 150 ml 95% ethanol, 2.0 ml 1 M sodium acetate, 1.4 ml 1 M acetic acid, and 45 g glycine per liter. The final pH was 5.50. Solution II contained 160 ml 95% ethanol, 2.5 ml sodium glycinate buffer pH 9.5, 3.2 ml 0.5 M disodium hydrogen phosphate, 2.4 ml 0.5 M sodium dihydrogen phosphate, and 45 g glycine per liter. The final pH was 6.8–6.9.

Sera: Sera from patients with *S. mansoni* infection were obtained from native Puerto Ricans; sera from patients with *S. hematobium* infection were supplied by the Naval Medical Research Unit No. 3 in Egypt; sera from patients with *S. japonicum* infection were obtained from U. S. Army personnel infected while serving in the Philippines. The diagnosis in all cases was established by the demonstration of parasite eggs in the stools. The syphilitic sera and those representing other infections were selected from specimens submitted to this laboratory for serologic examination; all were from well documented cases in which contact with schistosomiasis was extremely unlikely. The sera designated as healthy were from West Point candidates who had passed a rigorous physical examination.

METHODS

Antigen Isolation: Antigen was isolated from adult *S. mansoni* by combining extraction with

Na DOC, and fractionation by Method 10 of Cohn et al.[8]

Preliminary extraction: A 100 mg quantity of lyophilized adult worms was soaked for 1 hour in 12 ml of Na DOC, ground for 5 minutes in a glass tissue grinder with a motor driven Teflon pestle, extracted 4–6 hours, and finally ground again for 5 minutes. The suspension was then transferred to a stoppered tube and gently shaken for 10 minutes with 10 ml of chloroform-methanol (4:1). This extraction was usually permitted to continue overnight at 3–5°C. Centrifugation for 30 minutes at 1000 G separated the contents of the tube into three distinct layers; chloroform, debris, and aqueous. The antigen-containing aqueous layer was removed by aspiration, transferred to Visking cellophane tubing, and dialyzed for 72 hours against running tap water.

Purification processes: Two methods were used for isolating the serologically active antigen from the dialyzed solution.

Method 1. The antigen was isolated by fractionation with ethanol under conditions of carefully controlled pH and ionic strength. The dialyzed Na DOC extract was adjusted to pH 5.8 with 0.01 M acetate buffer, and cold ethanol added to a final volume of 16%. After standing overnight at −5°C, the precipitate was removed by centrifugation, and the supernatant discarded. The precipitate was stirred into a paste with a glass rod, and extracted for 1 hour at −5°C with 10 ml of cold solution I. The insoluble material was separated by centrifugation, and the inactive supernatant discarded. The sediment then was extracted as before, but this time using 15 ml of cold solution II for 2 hours at −5°C. The supernatant containing the active antigen was separated from the residue by centrifugation, and dialyzed first against running tap water for 24 hours to remove the glycine, and then against 0.85% sodium chloride for 12 hours to increase the solubility of the inactive components in the next step. The antigen was finally precipitated by adding 0.2 ml of 0.5 M calcium acetate and ethanol to 10% by volume. After standing overnight at −5°C, the slight precipitate, containing all the active antigen, was removed by centrifugation, washed once with a solution containing 20% ethanol and calcium acetate as above, dissolved in 0.85% sodium chloride and stored at −20°C. The antigen prepared by this method was designated as purified (P), and was used in serological and chemical tests.

Method 2. This method provided a relatively simple procedure for the preparation of an antigen for use in diagnostic tests. The antigen was precipitated directly from the dialyzed Na DOC extract by addition of 0.2 ml of 0.5 M calcium acetate, and ethanol to 10% by volume. After standing overnight at −5°C to assure complete precipitation, the antigen was sedimented by centrifugation for 30 minutes at 1000 G, then dissolved in 10 ml of 0.85% sodium chloride, divided into convenient amounts, and stored at −20°C.

Antigen prepared by either method and stored at −20°C retained its activity for over 6 months.

Complement fixation test: The method employed was a development of an earlier quantitative procedure described by Kent et al.[9] The reagent diluent was a triethanolamine-buffered salt solution (TBS)[10] containing Mg^{++} and Ca^{++} in concentration optimal for immune hemolysis.[11] The standardization of reagents and the test procedures were performed as previously described by Chaffee et al.[12]

Analytical procedures: Total nitrogen was determined by a micro-Kjeldahl adaptation of the method described by Hiller et al.,[13] protein by the method of Lowry,[14] and phosphorus by the procedure of Sperry.[15] The qualitative analyses for lipid and carbohydrate components were performed as spot tests on filter paper. The lipids were stained with Oil Red O,[16] cholesterol by an adaptation of the method of Schoenheimer and Sperry,[17] and carbohydrates by the fuchsin aldehyde test described by Koiw and Gronwell.[18] The antigen fractions were analyzed for purines and pyrimidines by paper chromatography using 254 mμ ultra violet light to examine the chromatograms.[19]

RESULTS

The method employed for extraction of the antigen determined the composition of the isolated material and hence its serologic activity. Antigens prepared by different methods were evaluated in complement fixation tests in the presence of a constant amount of antibody to determine the relative efficiency of various extraction procedures (Table 1). In each case, an antigen dilution of 1:1, was based on the concentration of antigen contained in 1.0 ml of the final product recovered from 10 mg of dried worms. The ether saline extract produced the most reactive antigen, but did not show inhibition in

the region of antigen excess. Antigen extracted with Coca's solution exhibited a similar reaction pattern, but the magnitude of reaction was significantly lower. Where Na DOC was the initial extractant, the method of purification influenced the serologic properties of the antigen component. For example, precipitation of the antigenic material by 80% ethanol resulted in a product that did not completely fix complement. On the other hand, antigen precipitated by calcium ion or isolated in fraction III-O exhibited similar reaction patterns, and showed marked inhibition of complement fixation in the region of antigen excess.

The serologic properties of fraction III-O (designated as P for purified) and the less pure ether-saline (ES) were evaluated in complement fixation tests with homologous and heterologous sera; the findings are summarized in Table 2. The ES antigen because of its stability and reactivity was chosen as a reference to provide a basis for evaluating the efficiency of the purification procedure. The syphilitic and normal sera were selected because of their reactivity with ES antigen. Evaluations were conducted in the region of gross antibody excess (undiluted serum) to provide a more critical test for cross-reactive components. It is noteworthy that both antigens were free from anticomplemetary activity. An important result of antigen fractionation was the marked reduction of cross-reactive properties. The less pure ES antigen gave strong reactions with the syphilitic sera, particularly in the area of antigen excess, whereas parallel tests with the P antigen were essentially nonreactive. Moreover, tests with homologous antisera revealed that the purification was achieved with essentially no loss of specific activity.

TABLE 1

Comparison of antigens prepared by different methods in their reactions with undiluted S. mansoni *antisera*

Antigen prep.†	Antigen diluted 1:						
	2.5	5.0	10	20	40	80	160
1. Ether-saline	0*	0	0	0	0	40	70
2. Coca's Sol.	0	0	0	5	35	75	100
3. Na DOC-80% alc.	5	10	10	10	20	70	100
4. Method 1.	45	10	0	0	10	75	100
5. Method 2.	40	10	0	0	5	70	100

* Numbers represent the percent hemolysis.

† Dried worms extracted with: 1. anhydrous ether followed by saline.[4] 2. Coca's solution.[3] 3. Na DOC precipitated by 80% ethanol.[6] 4. Na DOC fractionated by ethanol, precipitated by calcium ion. 5. Na DOC precipitated by calcium ion.

TABLE 2

Specific and cross-reactions of ether-saline (ES) and purified (P) antigens from S. mansoni

Antigen diluted 1:	Reactions* with human sera from											
	S. mansoni infection		*T. pallidum* infections						Healthy person		Antigen control	
	No. 1		No. 1		No. 2		No. 3		No. 1		ES	P
	ES	P	ES	P	ES	P	ES	P	ES	P		
2.5	4	3	4	±	4	±	3	±	1	−	−	−
5.0	4	4	3	−	4	−	2	−	−	−	−	−
10.0	4	4	1	−	3	−	±	−	−	−	−	−
20.0	4	4	±	−	2	−	−	−	−	−	−	−
40.0	4	3	±	−	1	−	−	−	−	−	−	−
80.0	2	1	−	−	−	−	−	−	−	−	−	−
160.0	±	−	−	−	−	−	−	−	−	−	−	−

* The recorded numerical values represent degree of complement fixation, the negative sign (−), no fixation.

Further differences of the antigens were manifested in titrations conducted to establish antigen optima. The results are given in Table 3. Titrations were performed by testing antigen, serially diluted in TBS, against antiserum diluted in non-reactive normal serum.[20] The P antigen showed the classic picture of inhibition in the region of antigen excess, thus permitting selection of an optimal concentration for the diagnostic test; *i.e.*, a concentration of antigen giving maximum reaction with threshold concentrations of antibody. The optimal dilution in this instance was 1:32. The fact that ES antigen exhibited no inhibition in the region of antigen excess suggests the presence of other antigens with overlapping but lower optima.

The antigens were evaluated further in tests conducted at the diagnostic level. Results of tests with homologous and heterologous sera are reported in Table 4. Neither antigen was species specific, as evidenced by their reactivity with *S. japonicum* and *S. hematobium* antisera. Of the 126 cases of verified *S. mansoni* infections, 113 reacted

TABLE 3

Standardization of ether-saline (ES) and purified (P) S. mansoni antigens for complement fixation tests

Antigen dilution		*S. mansoni* antiserum diluted 1:						Antigen control
Type	1:	4	8	16	32	64	128	
ES	2	4*	4	4	2	1	±	−
	4	4	4	4	2	1	±	−
	8	4	4	4	2	±	−	−
	16†	4	4	4	1	±	−	−
	32	4	3	2	1	−	−	−
	64	3	2	1	−	−	−	−
P	2	2	±	−	−	−	−	−
	4	3	1	−	−	−	−	−
	8	4	2	±	−	−	−	−
	16	4	3	±	−	−	−	−
	32†	4	3	1	−	−	−	−
	64	3	2	±	−	−	−	−

* The recorded numerical values represent degree of complement fixation, the negative sign (−), no fixation.

† The antigen dilution selected for diagnostic tests.

TABLE 4

Relative sensitivity and specificity of ether-saline (ES) and purified (P) antigens from S. mansoni

Diagnosis	Persons tested	Reactivity* with given antigen					
		ES			P		
		R	wr	−	R	wr	−
Schistosomiasis							
a. mansoni	126	112	6	8	85	28	13
b. japonicum	3	2	1			1	2
c. hematobium	9	7	1	1	3	2	4
Trichinosis	10		2	8			10
Paragonimiasis	1			1			1
Hookworm	6		1	5			6
Fascioliasis	1			1			1
Syphilis†	35	9	2	24			35
Healthy	50		1	49			50

* Classified as R (reaction 4+, 3+, 2+), wr (weak reaction 1+, ±), or − (no reaction).

† All strongly reactive in serologic tests for syphilis.

to some degree with P antigen, whereas 118 reacted with the ES antigen. The slight decrease in sensitivity of the P antigen, however, appears to be compensated by the increase in specificity. With sera from other infections, there were no reactions with the P antigen. The ES antigen, on the other hand, reacted with 15 of these sera. The findings suggest that purification removed the components responsible for the cross reactions with syphilitic antisera. The occurrence of several non-reacting *S. mansoni* antisera with both antigens may be explained by the fact that they represented early infections, and antibodies had not reached a detectable level.

The isolation of a second complement fixing antigen was made possible by substituting ether in place of chloroform-methanol in the treatment of the Na DOC extract. This second antigen precipitated during the dialysis of the Na DOC extract against water. An alkaline saline solution, pH 8, of the second antigen yielded a high titer (32) with the studied schistosomal antisera in the complement fixation test. However, it was not specific and had a low optimal dilution. Also, the calcium-precipitated antigen derived from the ether treated extract still contained non-specific components. For these reasons it was not studied further at this time.

In addition to serological analyses, the P antigen was subjected also to physicochemical analyses. Homogeneity was studied by free electrophoresis[21] and the agar diffusion technique described by Wilson.[22] In electrophoretic analysis, using veronal buffer pH 8.6 the P antigen migrated as a single component and showed no decrease in the height of the peak after 4 hours. When the antigen was tested in agar with homologous serum, using the double diffusion method, only a single precipitate band was found.

Qualitative analyses of the isolated P antigen suggest a lipoprotein structure. This corroborates the findings of Eliakim and Davies.[3] Elemental analyses yielded positive tests for protein, phosphorus, and lipid. It is noteworthy that no carbohydrate was detectable although it was present in the original extract. Paper chromatograms of an acid hydrolysate of the P antigen were negative for purines or pyrimidines when examined by ultraviolet light.

Comparison of the nitrogen and phosphorus content of the original extract and P antigen revealed the purification effected by fractionation (Table 5). The results were based on the amounts

TABLE 5

Quantitative nitrogen and phosphorus content of the original extract and purified (P) antigen

Product	Wt. worms, mg	μg N†	% N orig. ext.	μg P†	% P orig. ext.
Orig. extract*	10	500	100	30	100
P antigen	10	50	10	5	16

* Na DOC extract of dried worms, treated with chloroform-methanol, and dialyzed against distilled water.

† Average value of duplicate analyses on three different extracts.

of material present in undiluted antigen isolated from 10 mg of dried organisms. The P antigen contained 50 μg of nitrogen, or 10% of the original extract. With respect to nitrogen, this represented a tenfold purification over the original extract, while retaining maximum serologic activity. Moreover, information concerning the quantitative relationship between protein and lipid was estimated by utilizing the factors 6.25, to convert nitrogen to protein, and 25, to convert phosphorus to lipid. From these calculations the ratio of protein to lipid was found to be 2.5:1. Also, from these data the small quantities of antigen necessary for the standardized complement fixation test were ascertained. In diagnostic tests, using 0.3 ml of a 1:32 dilution of antigen, the amount of antigen involved would contain approximately 3 μg of protein and 1.2 μg of lipid.

DISCUSSION

Evaluation of various antigen preparations showed that the serologic activity of the derived product was determined to a large extent by the extraction process. Moreover, marked changes were effected merely by altering the order in which certain reagents were used. The substances extracted from *S. mansoni* by ether-saline, Coca's solution, or by 0.85% sodium chloride, though serologically active were not specific for schistosomiasis. Also, the cross-reacting components persisted despite numerous attempts to remove them by extraction processes or chemical fractionation. Only the Na DOC extraction procedure, of those tried, made possible the isolation of a specific antigenic component in which cross-reacting substances were undetectable. It was noted that fractionation of the Na DOC extract with ammonium sulfate, sodium chloride, combinations of saline and ethanol, or isoelectric precipitation produced antigens with relatively lower specific activities.

The chloroform-methanol treatment of the Na DOC extract contributed to the elimination of the non-specific substances, although the chloroform soluble material was only slightly antigenic in the complement fixation test. As previously mentioned, if ether was substituted for chloroform-methanol, a second antigen, which precipitated during dialysis, was isolated. Chloroform combined with amyl, isoamyl, or butyl alcohols in place of methanol produced no noticeable difference in the reactivity of the isolated antigen, nor did ether used in conjunction with chloroform-alcohol.

Method II evolved from Method 1, and offered a simple, direct procedure for the isolation of an antigen for use in diagnostic tests. The antigen isolated by Method II, while not always sufficiently pure for chemical analyses, was quantitatively as reactive in the complement fixation test as the P antigen (Table 1), and yielded similar serologic results in a number of parallel tests. The purity of this antigen during the calcium ion precipitation was determined to a considerable extent by the ethanol concentration. Any increases in ethanol beyond 10% resulted in concomitant precipitation of serologically inactive material. Dialysis of the precipitate was unnecessary as the presence of calcium had no deleterious effects on the complement fixing reaction.

The most pronounced improvement resulting from the purification of the antigen was the marked reduction of cross reactions with syphilitic sera (Table 2). The responsible substances were completely removed with only slight effect upon the reactivity of the antigen with homologous serum. In tests conducted at the diagnostic level (Table 4), the specificity of the P antigen again was demonstrated. No cross reactions occurred with any of the sera from patients with other helminthic or treponemal infections, whereas the ES antigen reacted to a considerable extent with syphilitic antisera and occasionally with other helminthic and healthy sera. Chaffee and Nieves[23] using ES antigen, similarly reported only slight cross reactivity with certain helminthic diseases; 2 cross reactions with 69 Puerto Ricans and none with 39 Trinidadians infected

with *Trichuris trichuria*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, and hookworm. On the other hand, Chaffee et al.[4] found no reactivity with syphilitic antisera using the ES antigen. The reason for this disagreement is not readily apparent. The three closely related infections, *S. mansoni*, *S. japonicum*, and *S. hematobium* were not differentiated on the basis of their reaction with either antigen. The findings suggest an antigenic similarity among the various species of schistosomes.

The selection of an optimal dilution, 1:32, for the P antigen was accomplished quite easily by titration due to the inhibition of complement fixing in the region of antigen excess (Table 3). This optimum compared favorably with the 1:16 dilution arbitrarily chosen for the ES antigen and the 1:20 used by Chaffee et al.,[4] and indicated that purification was achieved without loss of specific activity. This quantitative difference was noted in numerous preparations. Titrations employing other homologous sera yielded optimal dilutions of the same magnitude. The failure of the less purified antigens to exhibit inhibition due to antigen excess was attributed to the presence of other antigens with lower but overlapping optima. The increased qualitative reactivity of the less purified antigens also may be accounted for by a multi-antigen system, probably containing the antigen, precipitating during dialysis following an ether treatment of the Na DOC extract.

The lipoprotein structure of the P antigen was deduced from the chemical analyses of basic components. On the basis of quantitative nitrogen and phosphorus analyses (Table 5), the ratio of protein to lipid was found to be approximately 2.5:1. The protein and lipid moieties were closely associated as shown by the necessity of hydrolyzing the antigen before the lipid could be stained. Also, the serologic activity of the antigen seemed to be dependent on the presence of both the protein and lipid. The extraction of the lipid from the complex by the usual lipid solvents, alone or in combination with surface active agents, or by freezing with ether[33] caused a marked decrease of serologic activity. Heating the antigen for a few minutes at 100°C pH 6.5–7.4 resulted in coagulation with a concomitant reduction of activity in the complement fixation test. The P antigen was resistant to the action of the proteolytic enzymes, pepsin and trypsin, as are many conjugated proteins.

The solubility properties of the P antigen, including an isoelectric point around pH 5.4–5.6, suggested a possible globulin structure. Extraction into fraction III-O,[8] if analogous to human plasma fractions, implied a beta-lipoprotein or a lipid-poor euglobulin structure. The antigenic substance, however, differed from the human beta-lipoprotein, as described by Oncley et al.,[35] in that it contained no cholesterol, and its solubility and ultra violet absorption were dissimilar. Identification of the P antigen with a lipid-poor euglobulin would require further study.

The P antigen was not standardized on a dry weight basis because lyophilization resulted in a considerable loss in serologic activity.

SUMMARY

A complement fixing antigen, specific for the diagnosis of schistosomiasis, was isolated from adult *Schistosoma mansoni*. Following an initial extraction with sodium desoxycholate solution, the antigen was fractionated by ethanol under conditions of carefully controlled pH and ionic strength, and finally precipitated by calcium. The antigen was evaluated in a quantitatively standardized complement fixation test to determine its sensitivity and specificity, and was found to be more specific for the diagnosis of schistosomiasis than antigens isolated by other methods.

Qualitative and quantitative chemical analyses of the antigen showed the structural components to be protein and lipid, combined in the approximate ratio of 2.5:1. Attempts were made to relate the physicochemical properties of the antigen to its structure.

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NATURALLY ACQUIRED YELLOW FEVER IMMUNITY IN MUZO, COLOMBIA*

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Yellow fever was first diagnosed in the *Municipio* (County) of Muzo, State of Boyacá, Colombia, by Franco, Martínez-Santamaría and Toro-Villa[1] when they investigated an epidemic disease that was killing workers in 1907 at the world-famous emerald mines near the village of Muzo. The history of yellow fever at Muzo was reviewed briefly by Kerr and Patiño Camargo[2] and in greater detail by Soper.[3]

Kerr and Patiño Camargo (1933) found that 47 per cent of 88 specimens of human blood from persons resident in Muzo contained neutralizing antibodies for yellow fever; they concluded that yellow fever was endemic (we would now prefer the term "enzootic") in Muzo and in the surrounding country in the absence of *Aëdes aegypti*. Muzo, with its jungle yellow fever, is distant about 5,000 kilometers from the State of Espirito Santo, Brazil, where Soper *et al.*[4] first reported the condition.

The conclusions of Kerr and Patiño Camargo were based on sera from persons whose precise places of residence were not known. Age was known, but sex was not noted nor was length of residence in Muzo. The youngest positive was in the age group 10–14 years, in which 7 of 24 specimens, or 29 per cent, were positive. In 14 persons 40 years of age and over, 12 sera were positive, or 86 per cent.

In 1936 the studies here reported were begun in Muzo in order to determine the prevalence in the human population of immunity to yellow fever, as measured by the yellow fever neutralization test in mice, and its distribution by age, sex and length of residence prior to the use of any yellow fever vaccine in the study area.

This study was planned and initiated by Drs. Fred L. Soper and J. Harland Paul, of the staff of the International Health Division of The Rockefeller Foundation. One of us (MRG) was resident in Muzo for a period of over two years (1936–1938); another of us (JAK) succeeded Dr. Paul as Director of the Section of Special Studies in Bogotá, and was in turn succeeded by Drs. Hugh H. Smith and John C. Bugher.

Smith, Bevier and Bugher[5] published a brief summary of the results of the examination of 2,072 of these serum specimens, with 606 positive, 71 inconclusive and 1,395 negative results. The rules of interpretation they used are not stated but are presumably those of Sawyer and Lloyd.[6] The results are not analyzed by sex or length of residence but the percentage positive in children 0–14 years of age is 12.2, while in older persons it is 39.0. These indices follow the practice of Sawyer and Lloyd, which was to consider inconclusive results to be negative. Our interpretation is different. As will be explained later we consider inconclusive results to be positive, with the result that we find 14.7 per cent of children and 44.0 per cent of adults to be positive.

Description of Muzo—topography, climate and agriculture. The village of Muzo is located on the western slope of the Eastern Cordillera of the Andes at an altitude of 930 meters. A few miles to the west are the emerald mines, at an altitude of 687 meters. Between them runs the Rio Minero, at an altitude of more nearly 500 meters. These altitudes are typical of the whole *municipio* of Muzo, and indeed of the entire foothill area in which jungle yellow fever is a problem, extending from Caparrapi, Cundinamarca, on the south, some 400 kilometers north to Rio de Oro, Magdalena State. The width of the yellow fever area is from 25 to 50 kilometers and its altitude generally is in the range of 500 to 1000 meters. To the east of Muzo and the rest of the enzootic area the mountains rise to heights of 2500 and even 3000 meters—much too cold for yellow fever. To the west the land drops gradually to the Magdalena River, the banks of which are at about 150 meters above sea level in the latitude of Muzo.

The climate of Muzo is tropical and humid, with an average annual temperature of 23°C. The scanty rainfall data available indicate that

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the mean annual precipitation is about 3 meters, somewhat concentrated in a rainy season; there are only short dry seasons.

The agriculture of Muzo and the rest of the area is diversified, much of it at almost the subsistence level. The main cash crops are coffee and cocoa—the latter thriving only where the temperature and rainfall are favorable for jungle yellow fever. Cattle are produced in the region on pastures that are seeded after the forest has been cleared. Assorted food crops are grown, including maize, yucca, dry rice and sugar cane. Mining is not important in the area, even the emerald mines being worked only intermittently and often with imported labor. In the Magdalena Valley the oil fields are in the lowlands, where jungle yellow fever has not been a problem for reasons that are not entirely clear.

The climax vegetation of Muzo and the rest of the area is true tropical rain forest, most of which has been cleared so that the land can be used for agricultural purposes. Still left are much-abused patches of forest on slopes that are too steep to be worth clearing for farming. However, most of the usable timber has been removed from these patches. A second type of forest consists of the trees which are planted to shade the plantings of coffee and cocoa trees. The "forest" cover provided by these trees is much less dense than the other forest patches. The landscape of Muzo has a confused and untidy aspect. But this very untidiness provides a great variety of ecological niches, some of which are periodically, if not constantly, occupied by yellow fever virus in its extrahuman reservoir.

MATERIALS AND METHODS

As the first step, a census was taken of all the people living in the *Municipio* of Muzo, not only in Muzo village which has a population of about 350 but also in the 27 *veredas* (unofficial districts into which the *municipio* is divided). The name, age, sex, place (*vereda*) of residence and length of residence in the *municipio* were noted. A summary of these census data for 4,965 persons is given in Table 1.

A generous specimen of blood was then taken from as many of the residents as possible, using Bayer "Venules." These specimens comprised the "first bleeding." No specimens from suspect cases of yellow fever were included in these "survey" sera. At the base of the field unit in the village of Muzo, the blood serum was removed from the clot, using a small Venule, and eventually shipped to Bogotá. Ice was not then available in Muzo, and the specimens were not refrigerated in Muzo or after they reached the laboratory in Bogotá. The census-taking and the collection of the blood specimens began in February, 1936, and ended in May, 1937. Satisfactory neutralization test results were obtained on 2,129 serum specimens of the first bleeding.

Beginning in July, 1937 and continuing through July, 1938, a "second bleeding" was done of donors of first bleeding specimens in order to obtain evidence of serological conversions to yellow fever during the intervening period. Immediately after the second blood specimen was taken, the donors and all their families were vaccinated against yellow fever with the then newly available 17D vaccine. Satisfactory neutralization test results were obtained on 1,462 second bleeding specimens. Specimens from persons suspected of having yellow fever, or from contacts of such persons, were excluded from the second bleeding.

The examination of the sera by the intraperitoneal test of Sawyer and Lloyd[6] was done mostly in Bogotá at the laboratory of the Section of Special Studies, but several hundred sera were tested in the laboratory of the Yellow Fever Service in Rio de Janeiro, Brazil. The results obtained in Brazil were interpreted in the usual manner, except that when a serum which gave an "inconclusive" result in one test was retested, the result of the first test was considered to be just as valid as the result of the second; the results of both tests were used in making the final interpretation.

The great majority of the first bleeding sera tested in Bogotá were tested in mice that were not uniformly susceptible to yellow fever virus. The mice of the Swiss strain that had been brought from the United States when the Bogotá laboratory was started had been mixed with some mice of much lower susceptibility that were already in Bogotá. Although the proportion of relatively insusceptible mice was never determined precisely, it is believed to have been about 1 in 6. Many months elapsed before the mixed colony could be replaced with a new stock of susceptible Swiss mice from the United States.

In tests done with the mice of mixed stock, a larger-than-normal amount of virus was used in an attempt to reduce to a minimum the number of inconclusive results. The procedure was successful in the main, but it may have produced

TABLE 1

Summary of census of Muzo study area 1936–1937—town and 27 veredas

Length of residence	Age group (years)																								Total		
	0–4			5–9			10–14			15–19			20–29			30–39			40–49			50+			M	F	T*
	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T	M	F	T			
Whole life	392	426	818	329	306	635	269	218	487	169	190	359	248	328	576	179	180	359	112	107	219	63	82	145	1761	1837	3598
30 years plus																5	5	10	24	23	47	47	30	77	76	58	134
20–29 years													5	3	8	31	28	59	15	18	33	13	7	20	64	56	120
10–19 years										13	11	24	55	37	92	57	45	102	30	18	48	11	11	22	166	122	288
5–9 years							11	12	23	9	9	18	37	31	68	40	18	58	11	7	18	6	5	11	114	82	196
0–4 years				37	26	63	45	19	64	32	33	65	141	51	192	63	35	08	22	20	42	16	10	26	356	194	550
Total	392	426	818	366	332	698	325	249	574	223	243	466	486	450	936	375	311	686	214	193	407	156	145	301	2537	2349	4886

* Omitted from this table are 79 persons for whom the records were incomplete as to either age, length of residence or both.—Grand Total: 4,965

some falsely negative results. This possibility could not be examined in detail because the majority of the remaining portions of the sera were discarded once the negative results had been obtained. Nor could the donors be bled a second time, since many of them had been vaccinated against yellow fever by the time it was recognized that an unduly large amount of virus had been used in the neutralization tests.

The results of each neutralization test "run" were scrutinized carefully before the results were accepted as satisfactory. Numerous sera that gave positive results by the criteria of Sawyer and Lloyd were retested either in Bogotá or in Rio de Janeiro, and the positive result confirmed. Thus there is no reason to doubt the validity of the fully positive results.

Sera that gave inconclusive results by the criteria of Sawyer and Lloyd were all retested. The final interpretation was based on the cumulative results of both tests. If an inconclusive result was obtained on a serum that could not be retested, that result was considered to be unsatisfactory and was excluded from consideration in the final analysis.

While this procedure may have introduced an error into the results, the error is believed to be small; in any event it is well randomized through all the donors, by age, sex and length of residence.

In spite of the fact that the quality of the tests done on the first bleeding sera is known to be inferior to that of the tests done on the second bleeding sera, the same criteria have been used for the interpretation of the two groups of results.

After the elimination from consideration of all results interpreted as "unsatisfactory"—something quite different from "inconclusive"—all the Survival Ratios (SR's) of the results on each serum were added together and interpreted according to the following table:

Negative	*Positive*
0/4 1/4	2/4 to 4/4
0/5 1/5	2/5 to 5/5
0/6 1/6 2/6	3/6 to 6/6
0/7 1/7 2/7	3/7 to 7/7
0/8 1/8 2/8	3/8 to 8/8
0/9 1/9 2/9	3/9 to 9/9
0/10 1/10 2/10	3/10 to 10/10
0/11 1/11 2/11	3/11 to 11/11
0/12 1/12 2/12 3/12	4/12 to 12/12

It will be noted that this procedure qualifies the inconclusive results as "not negative," whereas the more common procedure is to consider them as "not positive." In our opinion, when one third (approximately) or more of the mice survive, the probabilities are that this survival ratio is due to the presence of antibody rather than to resistance of the mice or errors in technique.

The presence of yellow fever neutralizing antibody in the blood serum—according to the rules of interpretation just given—was accepted as proof that the person from whom the blood serum came had recovered from a yellow fever infection.

DIAGNOSED YELLOW FEVER IN MUZO—1936 TO 1938

In the years 1936 to 1938 yellow fever was diagnosed in Muzo, by laboratory methods, a total of 19 times, as follows: 1936: January—6 deaths, and May—2 deaths and 1 virus isolation; 1937: January—1 death, May—1 death, June—3 virus isolations, July—1 virus isolation, August—1 death and 1 virus isolation, October—1 death, and November—1 death. In 1938 there were no known cases of yellow fever; however, it may be recalled that the first yellow fever vaccination campaign in the *municipio* was not finished until July, 1938.

DISCUSSION

The results of the neutralization tests on the first bleeding specimens are set forth in Tables 2 and 3 and in Figure 1. Inasmuch as the results on persons who gave their residence as the village of Muzo, and were bled there, did not differ significantly from those of persons resident in the rural *veredas*, the data from the town and the 27 *veredas* have been combined in the tables and chart.

Among 708 males of all ages who were born in Muzo and were therefore considered to be lifelong residents, the proportion of positives for yellow fever antibody is 37.8 per cent. For 771 similar females, the proportion is 31.6 per cent. The rates for females are consistently lower than for males in all age groups over 0–4 years. In both males and females the proportion of positives increases rapidly with age up to age 30, but after that age remains fairly constant at nearly 70 per cent in the males and at about 60 per cent in the females.

TABLE 2

Results of yellow fever neutralization test—first bleeding—1936–1937, by age, sex and length of residence

Length of residence	Result	Grand total	Male									Female								
			Age in years								Total	Age in years								Total
			0–4	5–9	10–14	15–19	20–29	30–39	40–49	50+		0–4	5–9	10–14	15–19	20–29	30–39	40–49	50+	
Whole life	Number tested	1479	53	141	142	69	121	94	59	29	708	68	129	113	104	168	94	53	42	771
(Born in Muzo)	% positive	34.6	3.8	17.7	25.4	30.4	50.3	68.1	66.1	69.0	37.8	7.4	11.6	15.0	26.0	44.6	58.5	45.3	61.9	31.6
30 years plus	Number tested	73	—	—	—	—	—	5	15	28	48	—	—	—	—	—	2	15	8	25
	% positive	64.4	—	—	—	—	—	60.0	53.3	75.0	66.7	—	—	—	—	—	0	66.7	62.5	60.0
20–29 years	Number tested	69	—	—	—	—	7	12	8	8	35	—	—	—	—	6	12	13	3	34
	% positive	42.0	—	—	—	—	28.6	41.7	62.5	62.5	48.6	—	—	—	—	16.7	41.7	46.2	0	35.3
10–19 years	Number tested	187	—	—	4	6	36	40	16	4	106	—	—	5	12	22	27	10	5	81
	% positive	40.6	—	—	25.0	16.7	44.4	55.0	50.0	100.	49.1	—	—	20.0	16.7	31.8	22.2	50.0	60.0	29.6
5–9 years	Number tested	103	—	6	7	5	19	16	5	2	60	—	3	6	4	15	10	3	2	43
	% positive	22.3	—	16.7	0	0	36.8	37.5	20.0	50.0	26.7	—	33.3	0	25.0	6.7	20.0	33.0	50.0	16.3
0–4 years	Number tested	218	1	11	19	17	67	24	7	4	150	3	7	5	12	21	15	2	3	68
	% positive	17.4	0	9.1	0	5.9	22.4	33.3	28.6	25.0	18.7	0	0	20.0	8.3	19.0	20.0	0	33.3	14.7
Total	Number tested	2129	54	158	172	97	250	191	110	75	1107	71	139	129	132	232	160	96	63	1022
	% positive	34.0	3.7	17.1	21.5	23.7	40.4	56.5	57.3	69.5	37.4	7.0	11.5	14.7	23.5	37.9	44.1	47.9	59.2	30.5

TABLE 3

Results of yellow fever neutralization tests—first bleeding. Persons born in Muzo only, by year of age, and by sex

Age in years	Males				Females			
	Number tested	Number negative	Number positive	Per cent positive	Number tested	Number negative	Number positive	Per cent positive
1					1		1	100.0
2	7	7	0	0	11	10	1	9.1
3	21	20	1	4.8	20	19	1	5.0
4	25	24	1	4.0	36	34	2	5.6
5	24	23	1	4.2	25	24	1	4.0
6	33	26	7	21.2	25	23	2	8.0
7	31	25	6	19.4	24	20	4	16.7
8	35	29	6	17.1	40	36	4	10.0
9	18	13	5	27.8	15	11	4	26.7
10	33	28	5	15.2	27	19	8	29.6
11	14	11	3	21.4	15	15	0	0
12	45	35	10	22.2	32	28	4	12.5
13	28	17	11	39.3	19	18	1	5.3
14	22	15	7	31.8	20	16	4	20.0
15	16	14	2	12.5	24	18	6	25.0
16	13	7	6	46.2	19	13	6	31.6
17	10	7	3	30.0	15	12	3	20.0
18	22	14	8	36.4	33	24	9	27.3
19	8	6	2	25.0	13	10	3	23.1
20	21	12	9	42.9	29	15	14	48.3
21	13	9	4	30.8	9	4	5	55.5
22	15	8	7	46.7	28	17	11	39.3
23	12	6	6	50.0	11	4	7	63.6
24	7	2	5	71.4	7	6	1	14.3
25	27	11	16	59.3	35	17	18	51.4
26	7	1	6	85.7	10	5	5	50.0
27	7	3	4	57.1	10	6	4	40.0
28	12	8	4	33.3	23	14	9	39.1
29					6	5	1	16.7
30	16	2	14	87.5	23	14	9	39.1
31	8	2	6	75.0	7	4	3	42.9
32	14	5	9	64.3	8	5	3	37.5
33	3	1	2	66.7	4	1	3	75.0
34	1	0	1	100.0	3	2	1	33.3
35	25	11	14	56.0	23	5	18	78.3
36	12	4	8	66.7	9	1	8	88.9
37	4	2	2	50.0	5	1	4	80.0
38	8	2	6	75.0	12	6	6	50.0
39	3	1	2	66.7				
40	22	10	12	54.5	18	11	7	38.9
41	8	3	5	62.5	3	1	2	66.7
42	4	0	4	100.0	3	1	2	66.7
43	2	0	2	100.0	3	1	2	66.7
44	1	0	1	100.0				
45	13	5	8	61.5	15	7	8	53.3
46	6	1	5	83.3	5	4	1	20.0
47	1	0	1	100.0	3	2	1	33.3
48					2	1	1	50.0
49	2	1	1	50.0	1	1	0	0
50+	29	9	20	69.0	42	16	26	61.9
Total	708	440	268	37.8	771	527	244	31.6

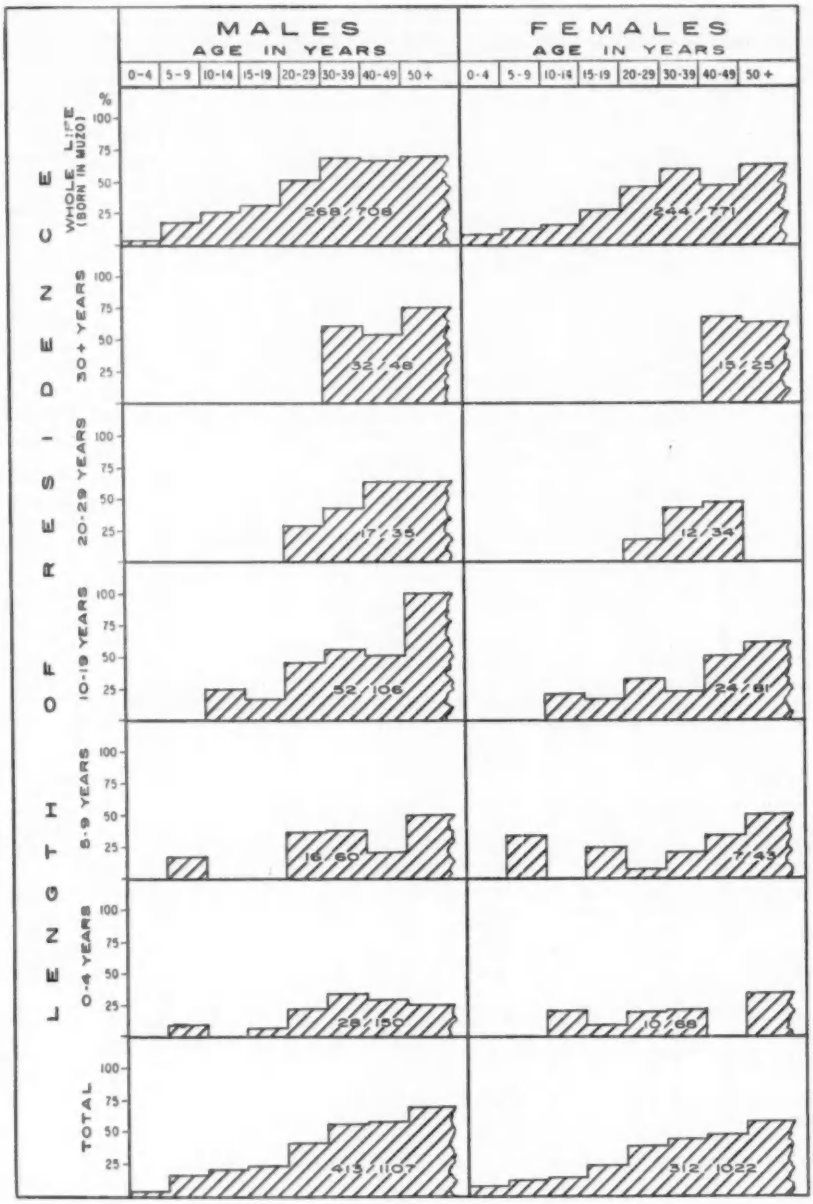

FIG. 1

The census gave a total of 3,598 persons born in Muzo; the weighted average per cent positive for this group is 29.4 per cent. The lower weighted figure is obviously due to the smaller-than-average proportion of young children bled and tested.

As with age, so with length of residence: for persons born outside the *Municipio* of Muzo, the

longer they lived in Muzo, the higher the proportion with antibody to yellow fever. The people who were bled in Muzo but had been born outside the *municipio* fall into two main groups: those born in the highlands where there is no yellow fever because of the cold, and those born in areas similar to Muzo where yellow fever is also present. Some of the latter probably already had yellow fever antibody in their blood when they moved to Muzo. No attempt has been made to analyze the results by the place of birth of persons born outside of Muzo.

The observed fact that females have lower rates than males in all age groups except 0–4 years, is consistent with a somewhat less intimate contact with the virus on the part of the females. While this circumstance would be readily understandable if the principal habitat of the virus were in the forest, the small differences between rates for males and females make it necessary to consider the possibility that much of the contact took place outside of the forest. The results show that contact with the virus begins early in life. Witness the fact that in the age group 10–14 years 21.5 per cent of all males and 14.7 per cent of all females are positive. These figures are noteworthy, since in many other jungle yellow fever areas in South America the percentage of positives in this age group is very small.

The serological conversions, and the reconversions as well, among the 1,462 persons bled twice are set forth in detail in Table 4 by age and sex. The crude, uncorrected conversion rate for males is 15.2 per cent, and for females, only 8.7 per cent. The rates for males are much higher in the older age groups than in the younger; for females the rates do not rise steadily with age, and there is no suggestion of a regular pattern.

TABLE 4

Serological conversion rates (net) on 1,462 persons bled twice, by age and sex

	Age group (years)								
	0–4	5–9	10–14	15–19	20–29	30–39	40–49	50+	Total
Males									
Total	45	124	138	61	136	119	68	38	729
Negative/Negative	42	111	105	52	79	48	27	12	476
Negative/Positive	3	5	18	6	17	20	10	6	85
Total negative	45	116	123	58	96	68	37	18	561
% conversions	6.7	4.3	14.6	10.3	17.7	29.4	27.0	33.3	15.2
Positive/Positive	0	5	13	3	38	48	28	20	155
Positive/Negative	0	3	2	0	2	3	3	0	13
Total positive	0	8	15	3	40	51	31	20	168
% reconversions	0	37.5	13.3	0	5.0	5.9	9.7	0	7.7
Net negative/positive	3	2	16	6	15	17	7	6	72
%	6.7	1.7	13.0	10.3	15.6	25.0	18.9	33.3	12.8
Females									
Total	65	119	105	88	152	114	59	31	733
Negative/Negative	61	104	87	70	111	67	36	19	555
Negative/Positive	1	7	12	8	9	10	5	1	53
Total negative	62	111	99	78	120	77	41	20	608
% conversions	1.6	6.3	12.1	10.3	7.5	13.0	12.2	5.0	8.7
Positive/Positive	2	6	5	10	28	37	15	10	113
Positive/Negative	1	2	1	0	4	0	3	1	12
Total positive	3	8	6	10	32	37	18	11	125
% reconversions	33.3	25.0	16.7	0	12.5	0	16.7	9.1	9.6
Net negative/positive	0	5	11	8	5	10	2	0	41
%	0	4.5	11.1	10.3	4.2	13.0	4.9	0	6.7

If the reversions are subtracted from the conversions and the resulting net conversion rates calculated, the same general picture is obtained, at an appreciably lower level of activity: 12.8 per cent for males and 6.7 per cent for females.

In a consideration of the significance of the conversions and the reconversions, it is to be remembered that according to the rules of interpretation of the Survival Ratios a 3/12 result is negative, but a 4/12 result is positive. Similarly a 3/6 result is positive, but a 2/6 result is negative. According to the laws of chance the variation is greatest in sera which do not have sufficient antibody to neutralize all the virus and produce fully positive results. Such a serum is much more apt to give a 5/6 result in one test and a 2/6 result in the next than is a serum that has sufficient antibody to give a 6/6 result in the first test. With a serum of the latter type a 2/6 result in a succeeding test of equal severity would be very unlikely. We have therefore treated the reconversions as a measure of the random scatter of the results and deducted them from the conversions. Very few of the reconversions were from 6/6 to 0/6, though there were some such episodes. At the time these neutralization tests were done it was not the practice to test both sera of a pair in the same run (*i.e.*, at the same time under conditions as nearly identical as possible).

The net conversion rates were analyzed in another way: by the interval between bleedings. It is to be expected that when a disease is present in an area, the longer the interval between tests, the higher will be the incidence of conversion. It was found in the present instance, however, that the conversion rates were appreciably higher in persons bled after about one year than in those bled after nearly two years. We conclude from this paradoxical result that variations in the incidence of yellow fever from *vereda* to *vereda* were much more important in determining rate of conversion than either the time interval between the two bleedings or differences in the sensitivity of the neutralization tests.

Although these results establish the fact that yellow fever is present in Muzo much of the time, they do not tell us whether the virus is constantly present in the *municipio* or whether it invades the *municipio* at frequent intervals. Certain information from other sources is pertinent. Gast[7] presents convincing evidence from the analysis of 594 proven deaths from yellow fever in Colombia during the 23-year period 1934 to 1956, that there is a marked periodicity in the invasion of certain areas by yellow fever. Other studies have revealed the presence of the virus in the *Municipio* of Muzo in every month of the year. However, along the right side of the Magdalena Valley the tendency is for the greatest number of cases to occur in the months of January and July.

Stated in another way, the problem is whether the virus is enzootic (permanently present) in Muzo or epizootic (present only intermittently). The terms enzootic and epizootic are used because jungle yellow fever is involved, and it is a zoonosis. It is desired to reserve the words "endemic" and "epidemic" for urban yellow fever, which is transmitted directly from man to man by *Aëdes aegypti*.

The histograms in Figure 1 give no evidence of periodicity—indeed the method of charting would mask any such evidence even if it were present. To study this matter further, three-year moving averages were calculated of the percentage of persons positive up to age 50. The calculations were made from the data in Table 3; they were made separately for males and females and only for persons born in Muzo. The results are shown in Figure 2.

Even though there is no clear evidence of periodicity in these results, they are presented to emphasize the fact that a great deal remains to be learned about the way in which human infections with jungle yellow fever are acquired.

A bit of historical perspective may be useful. Not until several years after these studies were completed was it recognized that more than one species of *Haemagogus* is present in the jungle yellow fever areas of Colombia. Taxonomic studies have revealed that there are three species: *H. spegazzinii*, *H. equinus* and *H. lucifer*.

In Muzo, *Haemagogus* mosquitoes have been taken biting man just outside of houses that were not in the jungle, and even inside houses. Thus the transmission of "jungle" yellow fever directly from man to man outside the jungle and even inside houses appears to be possible.

Monkeys are so rarely seen in the settled parts of the *Municipio* of Muzo that it seems probable that they have been exterminated. One must be a bit cautious about the night monkey, *Aotus trivirgatus*, because it is so elusive. Marmosets

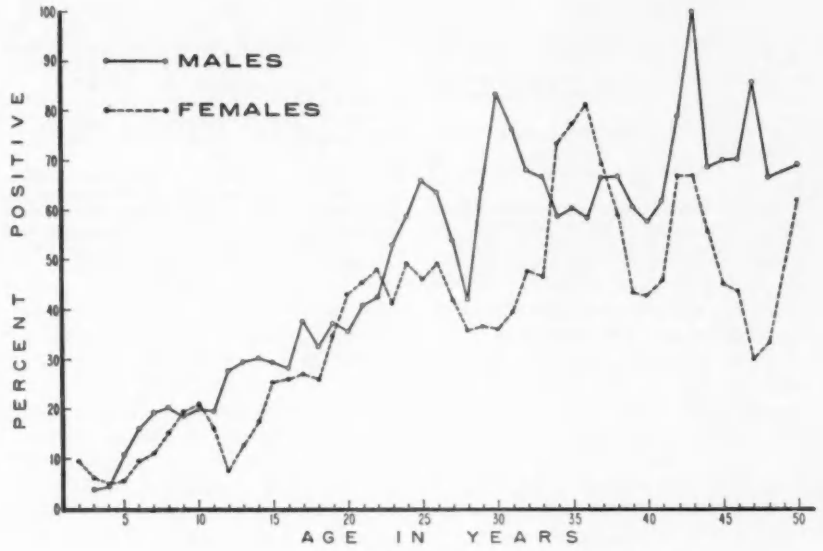

FIG. 2

are believed never to have been present in Muzo. Thus yellow fever persists in Muzo—and in much of the similar country that is subject to epizootic yellow fever—in the absence of wild primates. But there are still plenty of wild primates, of at least four different species, in the forested lowlands between Muzo and the Magdalena River. It is considered probable that jungle yellow fever is truly enzootic in this low-altitude area along the Magdalena River in central Colombia.

SUMMARY

In the years 1936 to 1938 a study was made of the immunity to yellow fever of the human population in the *Municipio* of Muzo, a typical jungle yellow fever area in the State of Boyacá, Colombia. Census data were taken on 4,965 persons, and two separate collections of serum specimens were made prior to the use of 17D vaccine in the *municipio*.

In the "first bleeding," 2,129 blood specimens were obtained. Because the mice used in the neutralization tests in the early part of the study were not uniformly susceptible to yellow fever, an excessive amount of virus was employed, and as a consequence special rules had to be set up for the interpretation of the test results; the most important of these was that inconclusive results were interpreted as positive. On this basis, the crude percentage positive of the first bleeding sera was 34.0, and that of 1,479 persons born in Muzo, 34.6. The proportion of positives increased with age and length of residence in Muzo, but there was an unexpectedly high percentage of positives in the 10–14 age group. While the number of positive males was appreciably larger than that of females, especially in the older age groups, the proportion of female positives to male was relatively high and suggests that transmission of yellow fever may not infrequently occur outside of forests. This hypothesis is supported by the observation that *Haemagogus* spp. have been taken biting well outside the forests and even within houses.

One to two years after the first bleeding, a second bleeding was done on 1,462 of the original donors. Net conversion rates (conversions less reconversions) for males and females were 12.8 per cent and 6.7 per cent, respectively. As would be expected, the rates were highest in adult males. In females the pattern by age group was irregular.

The data do not permit a conclusion as to whether yellow fever is enzootic or epizootic in Muzo.

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INTESTINAL NEMATODES IN WELL-CARED-FOR DOGS*

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Interest in the nematode parasites of dogs has been stimulated by new knowledge of their relations to human disease. Since its first discovery and identification in the liver of children[1] the larval stage of *Toxocara canis* has been observed in the tissues of a number of children,[2] and has been shown to be a cause of the disease known as visceral larva migrans.[3-5] Larvae of hookworms, *Ancylostoma braziliense* and *A. caninum*, long recognized as causing troublesome eruptions of the skin, are now known to invade the deeper tissues and reach the lungs.[6] The whipworm, *Trichuris vulpis*, likewise has recently been recognized as capable of development in man.[7]

Surveys in different parts of this country and elsewhere have shown that *Toxocara*, hookworm and whipworm infections all are widely distributed and generally are found in a high percentage of dogs, especially in the younger animals.[8] In most instances, however, the survey samples have been taken from the abandoned or poorly-cared-for class of dogs.

The present study was designed to determine the prevalence of intestinal nematode parasites in the well-cared-for class of dogs in the New Orleans area. Two approaches were made: In a university section of the city where property values are high and the general character of the neighborhood would indicate that most resident dogs are well cared for and homeless dogs are not tolerated, dog stools were collected from lawns and parkways and examined for helminth eggs. Following this phase of the study, stools were examined from dogs brought to veterinarians for various services, all relatively expensive and thus an indication of concern for the dogs' well-being.

METHODS

Searching both sides of the public sidewalk, a portion of a previously mapped-out section of the study area was covered on foot in early morning, usually between 7 a.m. and 9 a.m., samples being taken from all fresh dog stools observed. Scouting was limited to the areas observable from the sidewalk and no area was visited more than once. The study area covered approximately 100 residential blocks and the collections were made in June and July, 1956. Samples were examined by direct smear and by zinc sulfate centrifugal flotation. Egg-counts were made on standard direct smears containing 1 mg feces.[9] There were 170 specimens collected and examined in this manner (Figure 1).

Through the cooperation of the New Orleans Veterinary Medical Association, fecal specimens were obtained randomly from dogs admitted to four small-animal hospitals for treatment or for boarding during September, October and November, 1957. At the same time, clinical and environmental histories were obtained for each dog examined. There were 325 specimens obtained in this manner, each examined by direct smear, zinc sulfate flotation and formalin-ether sedimentation.

No attempt was made by culture or otherwise to make species identification of the hookworm eggs. It is presumed that essentially all were *A. caninum* since no other species has been found by autopsy of dogs in this immediate area.

RESULTS

Findings by the two methods of sampling were in close agreement (Table 1). Among the stools promiscuously deposited near the sidewalk, *Toxocara* was found in approximately 8%, whipworm in 16% and hookworm in 52%. Corresponding percentages found in the hospital group were 6, 19 and 46. Sex-age distribution of positive findings on the first group of course was not obtainable. In the second group, *Toxocara* infections were markedly more prevalent in the younger animals, the comparative percentages being 15% and 3% respectively in the sexually immature (under about 9 months) and mature dogs. The relative prevalence was reversed for

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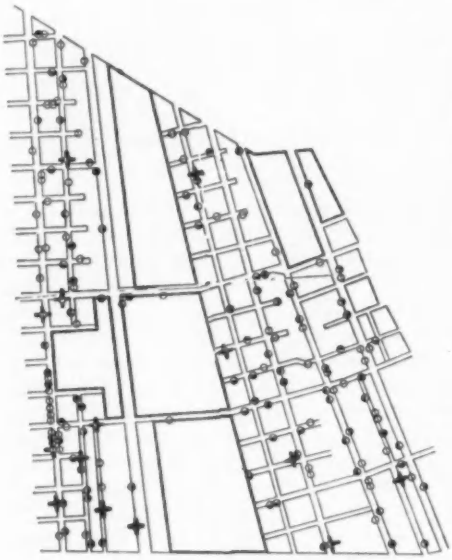

FIG. 1. Residential area on either side of university campus where dog stools were collected along public walks. Open circles represent negative stools; circles half-closed in the block indicate hookworm, those half-closed in the street indicate whipworm, closed circles indicate both hookworm and whipworm, and the crosses indicate *Toxocara* infections.

TABLE 1

Incidence of intestinal nematodes in New Orleans dogs admitted to veterinary hospitals and in dogs' stools collected from near the sidewalk in an exclusive neighborhood

Stools from	Near sidewalk	Veterinary hospitals		
		Young	Mature	All
No. examined	170	103	222	325
Per cent with:				
Toxocara	8.2	14.6	2.7	6.5
Hookworm	51.8	41.7	47.3	46.0
Whipworm	15.9	6.8	24.3	19.0
None	29.4	54.4	41.0	45.2

the whipworm, it being found in 24% of the older as compared with 7% of the immature dogs. Hookworms were found in approximately half of the animals in both age groups (Table 1).

None of the *Trichuris* or *Toxocara* infections in the sidewalk group was heavy enough to produce more than 20,000 eggs per gram of feces; two-thirds of them gave counts below 5,000 eggs per gram. The hookworm infections on the other hand were equally distributed in the light (under 5000/g), medium and heavy (over 20,000/g) classes. Similarly classified, the hospital group showed the *Toxocara* infections to be two-thirds light, one-third moderate; *Trichuris*, one specimen heavy, and the others equally divided between light and moderate; hookworm, two heavy, and the remainder two-thirds light, one-third moderate.

No significant differences could be demonstrated between the three infections with reference to host-sex. There was also only slight if any correlation between the incidence of infection in dogs confined to the house (except for exercise) and those allowed yard or neighborhood freedom, the percentages of infection being 48% of 32 confined dogs and 58% of 147 that were allowed some degree of outside freedom.

DISCUSSION

In an autopsy survey of over one thousand abandoned dogs in the New Orleans area *Toxocara*, *Trichuris* and hookworm infections were found in approximately 2%, 57% and 42% respectively.[10] These percentages being not markedly different from those found in the present study, it is apparent that the protection and treatment given pet dogs in this area are relatively ineffective in controlling the *Toxocara* and hookworm infections, and probably have little influence on the prevalence of whipworm infections. While in the present survey of dog stools deposited along the public walks the distribution of negative stools was somewhat spotty, the area distribution of the positive stools was more or less continuous, making it readily possible for infections to be acquired or reacquired by dogs when either allowed to range or when walked on a leash a few blocks in any direction from their residences (Figure 1). Under these circumstances the occurrence and reoccurrence of intestinal nematodes in a high percentage of dogs can be expected in spite of regular and frequent anthelminthic treatment.

The area distribution of dog stools bearing helminth eggs is likewise a factor in the rate of exposure of young children to infection with these parasites. Even when stools bear relatively few eggs, their daily addition to the soil

tends to maintain large numbers of infective stages in the surface layer. Movement from place to place by rain-drop splash, water currents, insects and other forms of transport tend to place an abundance of infective eggs or larvae in unsuspected sites, as already observed in door-yard soil surveys in New Orleans[11] and in Georgia.[12] Thus, while the probability of children's acquiring infection with dog parasites generally is greater when infected animals are in the same household, damaging infections in children who have had no known contact with pets can readily occur. One such case of *Toxocara* infection has been reported.[13]

In the metropolitan area of New York City where suitable areas for exercising dogs are limited, a recent study[14] has shown that their stools tend to be found abundantly in those areas. However, possibly because conditions generally are less suitable for the development of infective stages than in the New Orleans study-area, relatively fewer of the samples contained helminth eggs. In 100 stools from each of five areas, the average percentages with *Toxocara*, hookworm and *Trichuris* eggs respectively were 2, 7 and 5. It is possible, however, that in certain localities where soil and other conditions are favorable during the warmer months, there is more transmission of infection both to dogs and to children than the above figures indicate. In any case, whether in New York or in New Orleans, or in other similar localities, the probability of infection would appear to be so great that for the protection of either the dogs themselves or the children who range over the same areas, the general public if made aware of the attendant health hazards might wish to seek acceptable prohibitions against voluntary and intentional contamination of neighborhoods and public areas by the canine members of urban populations.

SUMMARY

Examination of 170 fresh dog stools collected from near the sidewalks of approximately 100 residential blocks, searched but once, in an exclusive neighborhood of New Orleans revealed *Toxocara* eggs in approximately 8%, hookworm in 52% and *Trichuris* in 16%. The area distribution of the positive stools was more or less equal and continuous. Stools from dogs admitted to veterinary hospitals for relatively expensive services contained *Toxocara* in 6.5% (15% of immature, 3% of mature dogs), hookworm in 46% and *Trichuris* in 19%. These percentages are not markedly different from those found in an earlier survey of abandoned dogs from the same area. It is apparent that even in well-ordered neighborhoods there is ready transmission of intestinal parasite infections among dogs, and under the observed circumstances the possibility of indirect transmission of these infections to children would appear to be considerable.

ACKNOWLEDGMENTS

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PHLEBOTOMUS (SANDFLY) FEVER VIRUSES IN TISSUE CULTURE*

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Phlebotomus (sandfly) fever appears to be endemic in the Mediterranean, Middle East, and certain areas of Russia. Both American[10, 7] and Russian investigators[1, 12] have been concerned with the etiology and epidemiology of this disease. Sabin[11] described the isolation and propagation in mice of two antigenically distinct strains of virus obtained from the blood of patients clinically diagnosed as having sandfly fever. According to the geographical source of the patients the two viruses have been called Naples and Sicilian, respectively.

In 1952–53, as part of an epidemiological survey being conducted by the Department of Virology of the United States Naval Medical Research Unit No. 3 (NAMRU-3), 22 viruses, subsequently identified as either Naples or Sicilian types, were isolated from human blood samples by the intracerebral inoculation of infant mice.[12] Because of the problems inherent in using infant mice it was considered highly desirable to find more feasible methods for the isolation and propagation of these viruses and for studies concerned with their immunological reactions. Numerous tissue culture systems have been screened for susceptibility to these viruses. The present report is concerned with the propagation of both Naples and Sicilian type viruses in human and mouse kidney cell cultures.

MATERIALS AND METHODS

Virus strains. Three Naples and two Sicilian Phlebotomus fever types were used in these tissue culture studies. Each initial inoculum was prepared from infected mouse brains and designated as follows: Naples (Na SF) types—H 1952, 22 mouse passages; H 2183, 18 mouse passages; Na SF (prototype)† 48 mouse passages; Sicilian (Si SF) types—H 5202, 14 mouse passages; Si SF (prototype),† 30 mouse passages.

Stock mouse brain virus suspensions in pH 9.0 borate saline containing 0.5% bovine albumin were stored in CO_2 ice. Infected tissue culture fluids were mixed with equal quantities of 20% bovine albumin and stored in CO_2 ice.

Preparation of tissue cultures. Mouse kidneys were aseptically harvested from 4–6 week old Swiss albino mice, minced and washed free of debris and blood cells with buffered saline solution. The tissue fragments were then trypsinized at room temperature in the usual manner. Finally, the free cells were centrifuged and resuspended in a growth medium consisting of Medium No. 199[6] containing 15% heat-inactivated calf serum and antibiotics. Culture tubes were seeded with 1.0 ml of a suspension having a final cell count of 5×10^5/ml, and incubated in a slanted, stationary position at 35–37°C. The growth medium was changed 3 to 4 days following seeding. When the cell monolayers were confluent, the growth medium was replaced with a maintenance medium consisting of 0.5% lactalbumin hydrolysate and 2% calf serum in Earle's balanced salt solution. Uninfected monolayers were kept in satisfactory condition for 2 to 3 weeks by changing the maintenance medium every 4 or 5 days.

Human cell cultures in tubes and bottles were prepared from adult and infant kidney tissue obtained at autopsy or surgery and were supplied by Dr. G. D. Hsiung.

Virus assay. Methods for determining virus plaque endpoints (plaque-forming units) were essentially similar to those described previously.[3, 4] Virus LD_{50} determinations in mice were calculated according to the method of Reed and Muench.[9] Tissue culture TCD_{50} titers were calculated from cytopathic reactions obtained using 6 tissue culture tubes for each log dilution of virus. Mouse brain or tissue culture fluid inocula were routinely adsorbed to the cell monolayers for 2 hours at 35–37°C after which time the cultures were washed 3 times with Hanks'

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† Kindly supplied by Dr. A. B. Sabin.

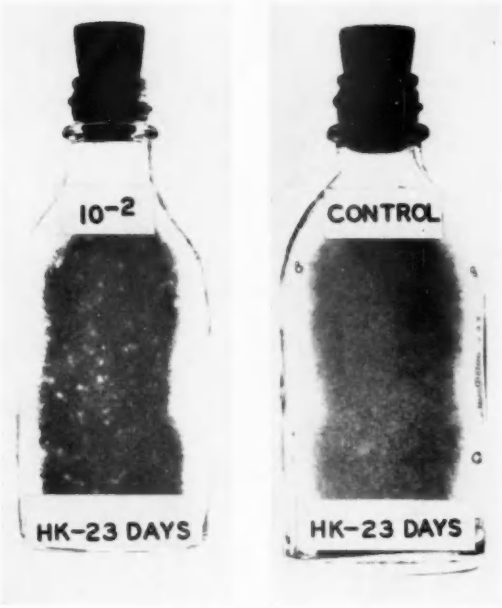

FIG. 1. Representative Phlebotomus fever plaques in human kidney monolayer bottle cultures. Left: H 1952 (Naples type), 10^{-2} dilution of virus, plaques appear 23 days after infection. Right: Uninoculated human kidney bottle culture.

balanced salt solution prior to the addition of maintenance medium or agar overlay.

RESULTS

Both the Naples and Sicilian types of Phlebotomus fever viruses multiplied in cultures prepared from human and mouse kidney origin. In human kidney agar overlay cultures, virus multiplication was manifest by the formation of plaques, only after a prolonged incubation period. The types of plaques formed after 23 days by a representative Naples type (H 1952) in human kidney cultures is illustrated in Figure 1. The titer of this virus as measured by the plaque method in human kidney cultures was $10^{3.2}$ PFU/0.1 ml; whereas, the endpoint in infant mice determined at the same time was $10^{5.9}$ LD_{50}/0.1 ml. Additional comparative titrations of other strains (H 5202 and H 2183) by the plaque method in human kidney cultures and infant mice yielded similar results, as shown in Table 1. Plaque titers in human kidney cultures were quite consistent regardless of whether the tissues were derived from infants or adults,

TABLE 1

Comparative titrations of Naples and Sicilian virus types in human kidney cultures and infant mice using infected mouse brain inocula

Virus strain	Human kidney ave. PFU/0.1ml*	Infant mouse LD_{50}/0.1 ml
H 1952—Naples	3.2†	5.9
H 2183—Naples	2.8	6.1
H 5202—Silician	3.5	6.6

* Six bottles per virus dilution.
† Log_{10} dilution.

but the sensitivity of the human kidney culture system to infection by the Phlebotomus fever viruses was apparently of a relatively low order compared with the titers obtained in infant mice.

The cytopathogenic effects (CPE) produced by representative viruses in human and mouse kidney cultures are illustrated in Figure 2. In human kidney cultures, CPE induced by both the Naples and Sicilian virus types were often not evident until the third or fourth fluid passage

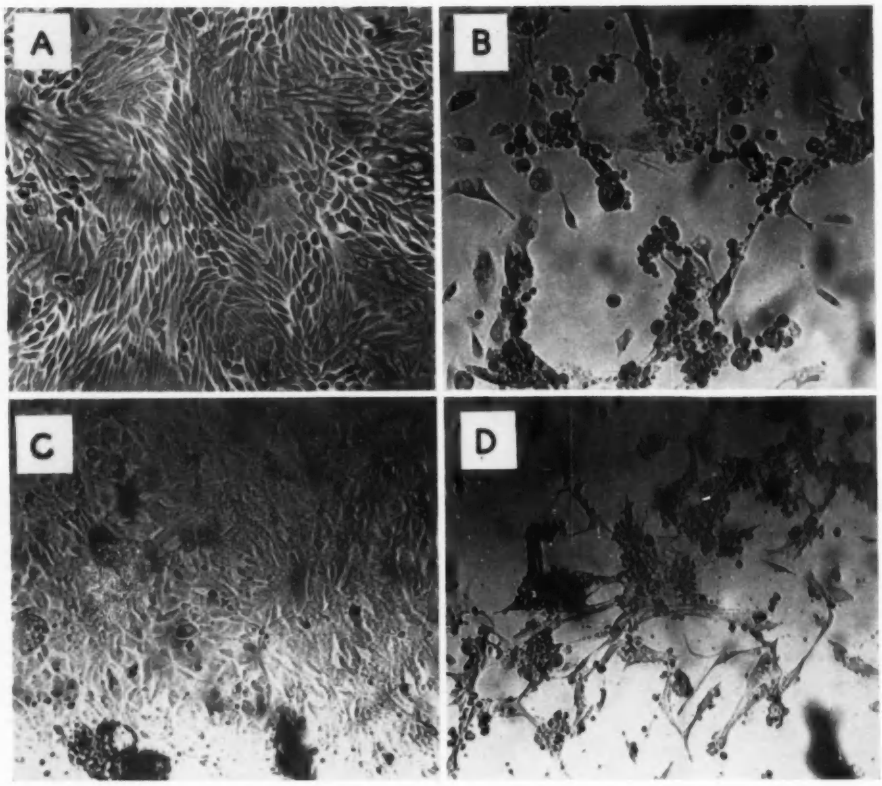

FIG. 2. Cytopathogenic effects (CPE) in human and mouse kidney tissue cultures by representative Naples and Sicilian virus types. A. Normal human kidney tissue culture, 8 days after seeding. B. CPE in human kidney tissue culture as the result of infection by H 2183 (Naples) virus, 3 days after virus inoculation. C. Normal mouse kidney tissue culture, 5 days after seeding. D. CPE in mouse kidney monolayer culture, 3 days after inoculation of H 5202 (Sicilian) virus.

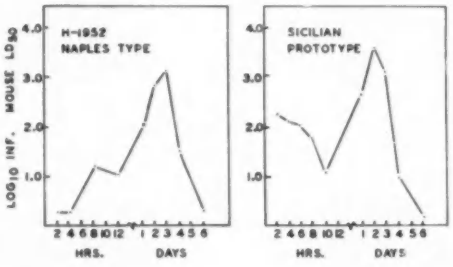

FIG. 3. Growth curves of representative Naples and Silician virus types in human kidney tissue cultures. Aliquots of the tissue culture fluids were titrated in infant mice over a period of 6 days.

or plaque subculture, and these effects could be demonstrated only in cultures prepared from infant kidney tissues. In mouse kidney cultures, however, CPE as a result of virus multiplication was usually observed in the first virus passage and occurred consistently with each of the virus types studied.

Growth curves of representative Naples and Sicilian viruses in human kidney tissue cultures are shown in Figure 3. Periodic harvests from fluid cultures were titrated in infant mice. Again, the efficiency of human cells to support multiplication of these viruses was of relatively low order. The peak virus titers were reached two to three days following inoculation with

TABLE 2

Comparative titrations of Naples and Sicilian viruses in mouse kidney tissue culture and infant mice using infected mouse brain as inocula

Virus strain	Mouse kidney TCD_{50}/0.1 ml	Infant mouse LD_{50}/0.1 ml
H 1952	5.0*	6.1
H 2183	4.5	5.7
Na SF	4.1	5.6
H 5202	4.6	5.5
Si SF	4.4	5.1

* Log_{10} dilution.

TABLE 3

Titrations of infected culture fluids from various virus passages in mouse kidney tissue cultures

Virus type	Mouse kidney tissue culture passage	Passage titration	
		Mouse kidney TCD_{50}/0.1 ml	Infant mouse LD_{50}/0.1 ml
Naples	2	3.5*	4.2
H 1952	4	4.4	5.3
	6	4.0	4.8
Sicilian	2	3.7	ND
H 5202	4	5.2	4.3
	6	5.6	4.5

ND—Not Done.
* Log_{10} dilution.

infected mouse brain virus. The growth curves of Naples and Sicilian virus types in mouse kidney cultures approximated those obtained in human kidney except that infant mouse LD_{50} titers of the infected mouse kidney culture fluids were generally higher. Preliminary data indicate that the infectivity half-life of both Naples and Sicilian virus types is about 6 hours, when incubated at 35–37°C in a cell-free tissue culture maintenance medium.

The mouse kidney culture system was more susceptible to infection than human kidney cultures and yielded virus titers almost equal to those obtained in infant mice. These data are shown in Table 2. Titrations of three Naples and two Sicilian strains were performed in mouse kidney tissue cultures and infant mice, in parallel. Each inoculum was prepared from the brains of infected mice. Generally, the first passage titers achieved in the mouse kidney cultures were about a log dilution lower than titers obtained in the infant mouse titrations. However, when each virus type at various tissue culture passage levels was titrated in mouse kidney cultures and infant mice, the results, as shown in Table 3, indicated that upon serial passage, the efficiency of the mouse kidney cultures to support virus multiplication increased for the Sicilian but not for the Naples type. In this experiment approximately 100 TCD_{50} doses of virus were inoculated at each tissue culture passage level, and after 48 hours aliquots of infected culture fluids were removed and titrated both in mouse kidney cultures and infant mice. The identity of the 6th tissue culture passage virus of each type was confirmed serologically.

DISCUSSION

Human kidney monolayer cultures were originally used for propagating the viruses of Phlebotomus fever in this laboratory, but the supply of such cultures was very irregular and almost prohibitive for use in extensive virus studies. Most of the human tissues available were obtained at autopsy, and difficulties were encountered when attempts were made to grow adult human kidney tissues removed 10–12 hours after death of the patient. Kidneys obtained from infants at autopsy could be grown in monolayers with relative ease even when the tissues were obtained 24 hours after death.[5]

It was desirable to find a susceptible tissue which could be easily grown in monolayer cultures and also which could be regularly obtained. Mouse kidney tissue proved to be satisfactory for these purposes. The yield of viable cells following trypsinization of mouse kidney was approximately 3.6×10^7 cells/gram kidney tissue harvested. Adequate culture material was constantly available for running consecutive experiments without intervening delays.

The variation in susceptibility of mouse and human kidney cultures to Phlebotomus fever viruses may have been influenced by the nature of the inoculum used. It is reasonable to expect mouse kidney cultures to be more sensitive to infection than human kidney cultures when using mouse adapted virus strains. We have some evidence to indicate that upon serial passage of either Naples or Sicilian virus strains in human

kidney cultures, the efficiency of these cultures to support virus multiplication increases to a limited extent with each successive virus passage.

The consistency of plaque formation and inconsistency of CPE in human kidney tissue monolayers is of considerable interest. The absence of CPE in fluid cultures of adult kidney may have been attributed to either the rapid healing or unrecognizable cytopathology of infected cells, as has been suggested previously.[8] We have encountered this phenomenon in other arthropod-borne virus-host cell systems[2] and for this reason have depended primarily upon plaque formation in agar overlaid tube and bottle cultures for detecting virus multiplication when CPE in fluid cultures were irregular or not evident microscopically.

SUMMARY

Propagation of the Naples and Sicilian types of Phlebotomus (sandfly) fever viruses in primary human and mouse kidney tissue cultures has been described. Plaques in human kidney cultures were delayed but were produced consistently by both Naples and Sicilian types. Cytopathogenic effects as a result of multiplication by either virus type, could be demonstrated in infant kidney cultures only after an adaptation period. In contrast, cytopathic changes could be seen in the first and subsequent virus passages when mouse kidney cultures were inoculated with either the Naples or Sicilian virus types. Mouse kidney cultures were found to be more susceptible to virus infection than human kidney monolayers.

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THE USE OF DITHIAZANINE AS A MASS TREATMENT FOR INTESTINAL HELMINTHIASIS

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McCowen et al.,[1] and Bueding and Swartzwelder,[2] demonstrated the effectiveness of dithiazanine iodide against animal and human intestinal helminths. Swartzwelder et al.,[3] indicated that the drug was effective for trichuriasis, strongyloidiasis, ascariasis and enterobiasis, with significant activity against *Necator americanus*, meaning that dithiazanine is the most effective broad spectrum anthelmintic. Similar conclusions were presented by Wagner et al.,[4] who also reported an apparent drug activity against two tapeworms, *Hymenolepis nana* and *Taenia sp.* Donoso and Donckaster[5] indicated that dithiazanine was not effective in the treatment of *H. nana* infections. Miller et al.,[6] found a cure rate of 100% for *Enterobius vermicularis*. Swartzwelder et al.,[7] reported a cure rate of 89% for strongyloidiasis using a 21 day treatment. Frye et al.,[8] reported the drug as a very effective trichuricide with cure rates of 71% and 87.5% depending on the dosage schedule. Studies performed in Colombia by Albornoz[9] showed good results for *Trichuris trichiura* and *Ascaris lumbricoides* infections and partial effectiveness for *N. americanus*. Most of the authors mentioned above reported some side-effects with the use of dithiazanine.

MATERIAL AND METHODS

We selected Barrio Castilla in the city of Medellín because it is a poor section with deficient sanitary conditions, is located close to the city and has a health center controlled by official sanitary authorities. Families were selected at random and one stool examination was performed by the Stoll quantitative method prior to treatment* in order to determine the degree of parasitic infection in each individual. After giving the drug to the infected persons and waiting for 4 to 6 days another stool examination was made by the same method to determine the effectiveness of the treatment. Intolerance to the drug was observed and noted. A group of patients infected with *Strongyloides stercoralis* were selected at Hospital de San Vicente, of this city, and a special control was performed in these cases. The patients were of both sexes and the ages ranged from 18 months to 68 years.

The following dosage schedule was used:

Under 15 kg of weight	200 mg daily
16–30 kg of weight	400 mg daily
Over 30 kg of weight	600 mg daily

The daily dosage was divided into two or three doses. All patients were treated for 8 days.

RESULTS

Initially, we studied 362 persons, 316 (87.2%) of whom were positive for helminths. A total of 273 persons were treated and examined after therapy. The number of persons with single or multiple infections were as follows:

With 4 helminths	6
With 3 helminths	35
With 2 helminths	134
With 1 helminth	98
Total	273

The four most common helminths found were: *T. trichiura*, *A. lumbricoides*, *S. stercoralis* and *N. americanus*. Some cases of *E. vermicularis* are not considered since no special examinations were performed for them.

Trichuris trichiura (*215 cases*). One hundred and forty-four (66.9%) became negative after therapy. In the remaining 71 cases there was a reduction in egg count of 71.9%.

Ascaris lumbricoides (*207 cases*). One hundred and sixty-nine (81.6%) became negative after treatment.

Strongyloides stercoralis (*45 cases*). Forty-two cases (93.3%) of strongyloidiasis were negative after treatment. In the 3 remaining cases the average number of larvae decreased from 5,800 to 1,270 (78.1% reduction). Duodenal drainage specimens were obtained from 17 cases after therapy and all were negative for larvae.

* The drug employed for this study was kindly provided by Eli Lilly and Company.

TABLE 1

Effectiveness of treatment with dithiazanine on intestinal helminthiases

Parasite	Total No. of infections	Negative after treatment	Still positive after treatment		
		% negative	No. still positive	Average pre-treatment egg count	% egg count reduction
T. trichiura	215	66.9	71	6,280	71.9
A. lumbricoides	207	81.6	38	19,296	81.1
S. stercoralis	45	93.3	3	5,800 (larvae)	78.1
N. americanus	41	41.4	24	7,171	65.9
Totals	508	73.2	136	9,637	76.4

Necator americanus (*41 cases*). Seventeen (41.4%) of the cases became negative.

The results are summarized in Table 1.

Signs and symptoms of intolerance to the drug appeared in 119 (43.5%) of the 273 patients treated. A good number of them were given uncoated tablets. The main complaints were referable to the gastrointestinal system. The manifestations were as follows: abdominal pain 46; vomiting 32; nausea 18; diarrhea 15; headache 8. In only 8 cases were the symptoms of vomiting and abdominal pain so severe that it was impossible to give the full treatment. Two of them had peptic ulcer; one had hepatitis and another one had congestive heart failure. In the rest of the patients the symptoms, which usually appeared in the first 2 or 3 days, disappeared spontaneously or with the administration of sedatives and antacids.

SUMMARY

In Medellín, Colombia, 362 persons were studied by means of stool examinations, and 87.2% of them were positive for intestinal helminths. A total of 273 persons, ranging in age from 18 months to 68 years, were treated with dithiazanine iodide with the daily dosage of 200 mg under 15 kg of body weight, 400 mg between 16 and 30 kg and 600 mg over 30 kg, all for 8 days.

The majority of the 273 persons treated had more than one species of helminth. The number of infections in the group was 508, caused by *Ascaris lumbricoides*, *Trichuris trichiura*, *Strongyloides stercoralis* and *Necator americanus*. Post-treatment examination revealed eradication of 372 infections, or 73.2%, cured. In the remaining 136 infections, the worm burdens were decreased considerably, for the average egg count per gram of feces was reduced from 9,637 to 2,280, i.e., 76.4%. Intolerance to the drug was observed in 43.5% of the cases. The reaction of cases ceased with simple treatment or spontaneously.

From this report the conclusion is drawn that dithiazanine iodide is effective for mass treatment of intestinal helminths common in Colombia.

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THE PROPHYLACTIC AND CURATIVE ACTIVITY OF 1-MALEINYL-4-(3′-CHLORO-4′-METHYL-PHENYL)-PIPERAZINE (HOECHST S 688) IN EXPERIMENTAL SCHISTOSOME INFECTIONS

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Since the discovery of Miracil D by Kikuth, Gonnert, and Mauss,[5] the oral activity of at least 3 new types of compounds against *Schistosoma mansoni* has been described. Schubert[9] fed infected mice relatively large amounts of certain thioantimonial compounds and rid the mice of worms. Raison and Standen,[7, 8] Standen and Walls,[10] Gorvin et al.[4] and Collins et al.[2] reported on the schistosomicidal activity of aminophenoxyalkanes and of related compounds following oral administration to mice and other rodents infected with *S. mansoni* and *S. japonicum*. More recently, de Meillon, England and Lammler[3] described the prophylactic and curative activity of the drug S 616 Hoechst in mice. Later, Lammler[6] indicated that the compound, 1 - maleinyl - 4 - (3′ - chlor - 4′ - methyl - phenyl) - piperazine sodium salt (Hoechst S688), had strong prophylactic and curative activity against the *S. mansoni* parasite (Liberian) in mice. In unpublished data (Hoechst Co.), the maximum dose of S 688 tolerated orally by mice was about 700 mg/kg body weight while monkeys tolerated about 200 mg/kg. The dose which cleared 50% of the *S. mansoni* infection in mice was 29 mg/kg and about 200 mg/kg in monkeys. In preliminary human trials in Africa, there were some minor side reactions to doses of 3–5 mg/kg given thrice daily for 10 days. Well-tolerated doses appeared to be insufficiently effective.

Samples of S 688 were supplied to these laboratories and the drug was tested for activity against experimental infections of *S. mansoni* (Puerto Rican) and of *S. japonicum* (Formosan).

MATERIALS AND METHODS

Mice and monkeys were exposed percutaneously to cercariae of Puerto Rican *S. mansoni* while dogs were similarly exposed to cercariae of Formosan *S. japonicum*. The Puerto Rican strain of *S. mansoni* was maintained in Puerto Rican *Australorbis glabratus* and in Swiss albino mice. The Formosan *S. japonicum* was maintained in albino mice and in *Oncomelania formosana* supplied through the courtesy of Dr. W. Dewitt of the National Institutes of Health and by Dr. H. Hsu of the University of Iowa. The exposures were made according to the following schedule: approximately 150–250 *S. mansoni* cercariae per mouse, 125–250 *S. mansoni* cercariae/kg body weight for the monkey, and about 25–50 *S. japonicum* cercariae/kg body weight of the dog.

Various regimens of the Hoechst drug were administered to the animals at different times during the course of infection. In the prophylactic tests, the drug was given orally on the day of exposure, or on one or more of the four days following exposure. Tests of curative effect of oral or parenteral injections of S 688 were started when schistosome eggs were found in the feces of the animals. Solutions of the drug were administered to mice by stomach tube or by hypodermic needle while the larger animals received the drug in enteric-coated tablets. At start of tests, about 5 mice, and frequently 10 or more, were placed on each regimen or were maintained as non-medicated controls.

Observations were made on the treated animals for signs of toxicity. Changes in body weight of the animals were recorded during treatment and reactions such as weakness, ataxia, and other signs of intoxication were noted. Samples of blood and urine were collected from some of the treated dogs. The urine samples were examined for the presence of sugar and albumen. Counts of red and white blood cells and determinations by hematocrit, and of hemoglobin, were made on the same dogs.

Two procedures were utilized in observing the activity of S 688 in experimental schistosomiasis. Before and after treatment, the feces of the larger animals on curative tests and their non-treated controls were examined for numbers of eggs by the direct smear method and the AEX or AMS

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III concentration techniques. A significantly greater reduction in eggs passed by treated animals over that by the controls was an indication of drug activity. However, the evaluation of curative efficacy was based on results of autopsy examination of the animals usually made 3 to 4 weeks after completion of curative treatment. The schistosomes remaining alive after treatment, were counted in the perfusates of the hepatic portal system and in press preparations of the liver. In the same preparations, the relative numbers of dead parasites and worm eggs in the liver were ascertained. The efficiency of each treatment was that percentage of the control infection represented by the difference between numbers of parasites recovered from treated and the non-treated groups. About 2 months after prophylactic regimen, the average worm burdens of the treated and non-treated control animals were compared and the protective value of the drug was determined.

RESULTS

Prophylaxis Test Results. Table 1 summarizes the prophylactic effects of S 688 observed in several groups of mice that were treated orally one or more times beginning at time of exposure to cercariae to 4 days after exposure. The groups of mice were usually divided into 3 lots, one received a dose of 72 mg/kg of the drug, the second a 120 mg/kg dose, and the remaining lot served as infection controls. The animals in each of these lots were exposed to cercariae at the same time and under the same conditions. Two months after treatment, the variations in the number of worms found in the mice are indicated in Table 1 as the standard error of mean infection of each group. Although there appeared to be some difference between the size of infection in the animals treated at the time of exposure and the non-treated controls, this difference was not significant in statistical test. However, significantly smaller worm burdens were found in the lots of mice which had received the drug either 6 hours after exposure or during any one of the 3 days following exposure. As can be seen in the table, the estimated percentage reduction in the infection increased from 22% to 92% as the time of drugging was delayed up to the 3rd day after exposure. The dose of S 688 given on the 4th day after exposure resulted in an estimated 39 and 51% reduction in infection but these reductions were not significant because of the relatively great variations observed in the worm counts in these animals. In general, it was found that the higher dose of 120 mg/kg was more strongly prophylactic than the lower doses given. The regimen of 3 doses of 60 mg/kg given once daily starting the day after exposure, completely protected one group of seven animals and only one schistosome was found in another similar group. The infection controls had an average of

TABLE 1

Prophylactic activity of compound S 688 (Hoechst) against Schistosoma mansoni *infection in mice*

Treatment at exposure—(0); after exposure (+) (days)	Average number schistosomes/mouse										
	Medicated groups									Non-medicated controls	
	60 mg/kg			72 mg/kg			120 mg/kg				
	No. mice	Av. No. worms/mouse	% Efficiency	No. mice	Av. No. worms/mouse	% Efficiency	No. mice	Av. No. worms/mouse	% Efficiency	No. mice	Av. No. worms/mouse
0				4	30.5 ± 10.3*	22.6	5	26.0 ± 6.4*	34.1	5	39.4 ± 8.2*
+¼				5	14.0 ± 6.2	45	5	5.6 ± 0.8	78.0	5	25.8 ± 1.6
+1	11	11.4 ± 3.5*	76.6							12	48.3 ± 3.1
+1				5	20.4 ± 3.6	50.3	5	8.6 ± 3.6	79.0	5	41.0 ± 3.8
+2				5	24.4 ± 7.6	41.9	5	12.0 ± 2.4	71.5	5	42.0 ± 4.4
+3				5	27.0 ± 5.4	66.7	5	6.4 ± 4.2	92.1	5	81.0 ± 5.0
+1, +2, +3	7	0.1 ± 0.1	97							7	45.3 ± 7.6
+1, +2, +3	7	0.0	100							10	7.1 ± 2.1
+4				4	33.0 ± 26.5	51.6	4	41.0 ± 18.0	39.9	5	68.2 ± 7.8

* Standard error of the mean.

45.3 worms each. These results indicate that a series of daily doses would probably completely protect mice against *S. mansoni*. This was the case in 5 mice which had received a daily dose of 50 mg/kg starting the day before exposure and continuing through the 3rd day after exposure.

However, the *S. mansoni* infection apparently developed normally in three monkeys after each had been given one of the following oral regimens of S 688: 100 mg/kg 24 hours after exposure; a dose of 240 mg/kg on each of the 3 days following exposure; or a dose of 125 mg/kg on each of 5 consecutive days starting on the day of exposure. At autopsy, a total of 194 and 202 schistosomes were found in the latter two monkeys while a non-treated control had 233 parasites.

A dog given a single dose of 50 mg S 688/kg 48 hours after exposure to *S. japonicum* cercariae and another which had received 25 mg/kg on each of the 2 days following exposure, became infected and passed schistosome eggs. No parasites were found in a third dog which received 3 doses of 25 mg/kg, one on each of the 3 days following exposure while 25 worms were recovered from the non-medicated control animal. Since the latter was a relatively light infection, the prophylactic value of the same 3-day regimen was tested again in two additional dogs. Two schistosomes but no eggs were found in the first animal while the second harbored 12 parasites and some eggs. However, 41 and 65 worms were perfused from the two non-medicated controls which also had many egg-containing lesions in the liver and colon.

Table 4 summarizes the clinical findings on three of the dogs. There is no evidence herein that there were abnormal changes in the blood nor loss of weight resulting from treatment. Likewise no sugar or albumen was found in the urine.

Curative Test Results. Eight groups of mice with mature infections of *S. mansoni* from 6 to 8 weeks old, were divided into lots and some were treated with different doses of S 688 and by different routes as indicated in Table 2. In 3 groups, some of the mice received the drug

TABLE 2

Summary of results obtained in chemotherapeutic tests with S 688 (Hoechst) against mature schistosome infections

Test	Animals			Regimen			Route	Autopsy day post treatment	Av. No. live worms/animal		% Efficiency of regimen
	No. treated	No. non-treated	Type	Dose mg/kg	Dose No./day	No. days			Treated	Non-treated	
1	9	10	Mouse*	50	1	5	Oral	19	6.5 ± 2.1‡	7.1 ± 2.1‡	0.0‖
2	20	18	Mouse	60	1	5	Oral	23	14.2 ± 1.8	29.5 ± 1.9	51.9
	15	18	Mouse	60	1	5	I.P.	23	26.1 ± 1.2	29.5 ± 1.9	0.0‖
3	5	4	Mouse	60	1	5	Oral	23	15.4 ± 4.2	38.0 ± 3.7	59.5
	4	4	Mouse	60	1	5	I.P.	23	36.0 ± 6.5	38.0 ± 3.7	0.0‖
4	4	8	Mouse	60	2	5	Oral	17	3.2 ± 1.7	31.6 ± 6.1	89.7
5	8	10	Mouse	72	1	1	Oral	28	5.4 ± 4.7	50.0 ± 1.6	89.2
	10	10	Mouse	120	1	1	Oral	28	5.2 ± 5.2§	50.0 ± 1.6	89.6
6	4	9	Mouse	120	1	1	I.P.	21	54.7 ± 5.0	42.8 ± 5.7	0.0‖
	4	9	Mouse	240	1	1	Oral	28	3.7 ± 0.5	42.8 ± 5.7	91.4
7	3	9	Mouse	500	1	1	Oral	17	1.0 ± 1.0	19.1 ± 2.8	94.8
8	9	9	Mouse	500	1	4	Oral	17	1.5 ± 0.4	32.0 ± 2.7	95.3
9	1	0	Monkey*	100	1	5	Oral	65	8.0	—	—
10	2	1	Monkey	240	1	5	Oral	21	0.5 ± 0.5	95.0	99.5
11	1	1	Dog†	10	1	5	Oral	79	105.0	144.0	0.0‖

* Infected with *S. mansoni*.

† Infected with *S. japonicum*.

‡ Standard error of the mean.

§ Live worms were found in only one of these animals.

‖ No dead schistosomes found in treated animals.

orally and others received it intraperitoneally. Either one-third or one-half of each group was maintained as untreated controls. Three to 4 weeks after treatment the average size of infection in the treated groups of mice was compared with that of the infection control groups. The efficacy of the different regimens was determined as in the prophylaxis series. Dosages of 50 mg/kg given orally and 60 mg/kg intraperitoneally for 5 consecutive days were ineffective but the latter dose given orally reduced the number of worms in 2 groups of mice to about half that found in the controls. Of eight mice receiving a single oral dose of 72 mg/kg, five animals had no living worms. The three positive animals had 1, 3, and 38 live worms. Nine of 10 animals receiving a single oral dose of 120 mg/kg had no living worms but the remaining mouse had 53 normal worms. These variations were considered as the standard error of the mean in the statistical analyses which indicated that those groups given an oral dose of from 72 to 500 mg S 688/kg harbored an average infection which was significantly smaller than that in their infection control groups. It was noted that by increasing the single dose from 120 to 240 mg/kg the efficiency of the oral treatment increased less than 2% or from 89.6% to 91.4%. The oral dose of 500 mg/kg improved the curative efficiency of S 688 to 94.8%. However, it is questionable whether this is a real increase in efficiency over that of 89.6%. The 500 mg/kg dose resulted in loss of weight and death in some of the infected mice. In the groups of mice in which large numbers of worms were killed, frequently many of the animals were successfully cleared of parasites and only a few individuals in each group had living worms and some of these had infections as large as in the non-treated control mice. No dead parasites were found in mice examined up to the 23rd day after treatment with one intraperitoneal dose of 120 mg S 688/kg. A few dead worms were found in a group of animals which had received an intraperitoneal dose of 240 mg/kg. Since there was evidence that some of the drug remained unabsorbed in the peritoneal cavity 23 days after parenteral injection and the appearance of the schistosomes was slightly abnormal, observations were made on the numbers of live parasites removed from mice at intervals from 7 to 51 days after treatment.

Studies of the rate of disappearance of living adults of *S. mansoni* in animals which had been treated with S 688 were conducted as follows. A series of 75 animals was divided into 3 lots, one of which was given an oral dose of 72 mg S 688/kg, another 120 mg/kg orally while the third lot received a single dose of 240 mg/kg intraperitoneally. A lot of 10 mice was maintained as untreated controls. Five of the mice in each lot were examined at weekly intervals after treatment. The average size of the infections found in these animals is indicated in Table 3. Although no dead worms were found at the end of the first week, by the end of the second week it was estimated that the number of live worms was reduced by 28% and 40% in the two lots

TABLE 3

Rate of action of S 688 on Schistosoma mansoni *in mice*

Autopsy day post treatment	Average number living worms/mouse						
	Medicated groups						Nonmedicated
	Oral				Intraperitoneal		
	72 mg/kg		120 mg/kg		240 mg/kg		
	Av. no. worms/mouse	% Reduction infection	Av. no. worms/mouse	% Reduction infection	Av. no. worms/mouse	% Reduction infection	Av. no. worms/mouse
7	59.5	0.0	61.5	0.0	63.4	0.0	60.0
14	43.0	28.0	36.0	40.0	59.7	0.5	60.0
21	11.5	80.8	21.5	64.2	59.4	1.0	60.0
28	5.2	91.3	5.1	91.5	64.0	0.0	60.0
51	1.1	98.2	1.1	98.2	0.0	100.0	60.0

TABLE 4

Examination of dogs infected with Schistosoma japonicum *and treated with S 688 (Hoechst)*

Oral treatment daily dose			Changes observed*										
			Body weight		RBC Count (millions/ cu mm)		WBC Count (thousands/ cu mm)		Hematocrit, %		Hemoglobin, gm/100 ml		Reactions to treatment
Dog	mg/kg	Days	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx	
1	25	3	20	21	5.93	5.62	10.95	10.8	46	46	13.0	13.6	None
2	25	3	10½	11	6.06	5.96	11.55	11.35	50	50	14.8	18.0	Emesis after last dose
3	0		12½	13	6.61	6.31	12.95	11.85	49	47	14.0	14.6	

* Observations made just before treatment and 3 days after last dose.

treated orally. These percentages continued to rise to 64% and 81% by the end of the third week; 91% at the end of the fourth week; and 98% at the termination of 51 days. The few live worms remaining in these last animals were abnormal in appearance. Practically no dead schistosomes were isolated from the mice up to 28 days after the drug was injected intraperitoneally but all the worms were found dead in the liver of the animals examined 51 days after this treatment. The ten untreated controls harbored an average of 60 live worms. Twenty of a second series of 29 infected mice were given a single oral dose of 240 mg S 688/kg. Five of the treated animals were examined at weekly intervals after treatment for a period of 4 weeks. The nine untreated control mice harbored an average of 66.8 worms. It was found that by the end of the 1st week after treatment, the living worms in the treated mice were reduced by 75% and by the end of the 3rd week 96% were destroyed. This percentage did not increase by the end of the 4th week.

Three monkeys with mature infections of *S. mansoni* were treated with S 688. One of these animals (*Cercopithicus aethiops*) received a dose of 100 mg/kg daily for 5 days while two (*Macaca irus*) received a dose of 240 mg/kg daily for 2 days. The latter dose caused the monkeys to be ataxic and to suffer convulsions. When the first monkey was examined 65 days after treatment, many dead and dying worms were found in the liver. Only eight active worms were present. The egg counts of the two given the larger doses dropped markedly during a 30-day post-treatment period. Then the number of eggs in the feces began to increase and a dose of 240 mg/kg was administered daily for 5 days. On the 8th and 13th days after the second course of treatment, the stools became negative and remained so until the animals were autopsied 21 days later. In one animal a single pair of disintegrating worms was found and the other monkey had four encapsulated worms in the liver, remnants of other worms, and a single abnormal male in a vein of the colon. Comparative egg and worm counts with the control animal and with many other infected monkeys indicated that the two treated animals originally had approximately 299 and 93 worms respectively. It is believed that if these two animals had been examined a week later no living worms would have been found.

In a dog with *S. japonicum* infection, there was no evidence of any curative effect of a regimen of 10 mg S 688/kg given orally once daily for 5 days.

DISCUSSION

The prophylactic and curative activity of S 688 against the Puerto Rican strain of *S. mansoni* is similar to that found by Lammler and co-workers in experiments with the Liberian strain of this parasite. Both strains apparently are more susceptible to the drug than the Egyptian strain. However, Lammler found the greatest prophylactic effect when a dose of the drug was given at the moment of infection or 2 to 4 hours later. In the present study maximum prophylaxis was obtained by giving the drug 6 to 72 hours after exposure to schistosome cercariae. The fact that Lammler injected cercariae into

his animals rather than allowing them to penetrate the skin could explain this difference. In the limited tests of prophylaxis in dogs and monkeys reported herein, there was only evidence that S 688 prevented some of the *S. japonicum* infection from developing in dogs.

The drug was found to exert strong curative activity against *S. mansoni* in mice. Single oral doses of 72 mg/kg and larger cleared the mice of most of the infection. The worms remaining alive at the time of examination, usually 3 or 4 weeks post-treatment, were weakened and may never have recovered. This possibility was indicated by the finding that worms continued to die in mice beyond the 28th day after a single oral dose of 72 or 120 mg/kg. This appeared to be the case with the intraperitoneal regimens since very few dead worms were found by the 28th day post-treatment but all the worms were found dead by the 51st day. Since the drug was slowly absorbed from the peritoneal cavity this may explain the delayed cure.

The chemotherapeutic results utilizing this drug in larger animals were not as promising. In the monkey, the 100 mg/kg dose of S 688 was not completely curative of the *S. mansoni* infection while the 240 mg/kg dose was curative but toxic. Doses of 25 mg/kg and higher produced emesis in dogs and a test of an oral regimen of 10 mg/kg once daily for 5 days failed to have any observable effect on an infection of *S. japonicum* in the dog. The observed variations in efficacy of S 688 may not only have been due to the differences in the schistosome infections in various hosts but also to species differences in disposition of the drug.

De Meillon et al.[3] reported the compound Hoechst S 616, which is 2-chlor-3,5-dimethyl-(4-diethylamino-ethylamino)-toluene hydrochloride, to be active against both the immature and the mature stages of *S. mansoni* in mice. This compound is similar to S 688 in that they both have a benzene ring with similar substituents but different side chains and are free of antimony. Both compounds are highly effective against schistosome infections in mice and may have a similar mechanism of action *in vivo*. An elucidation of the metabolism of these compounds might be of value in the development of a better chemotherapeutic agent for the treatment of schistosomiasis.

These compounds, S 616 and S 688, are the first non-antimonial compounds to have marked prophylactic and curative activity in experimental schistosomiasis. A comparison of the efficacy of S 688 found in this study with that obtained previously by the oral administration of the accepted drug, Miracil D, under similar test conditions indicated that S 688 was superior in mice infected with *S. mansoni*. However, in infected monkeys, the advantage of S 688 was not so apparent.

SUMMARY

Oral regimens of the compound 1-maleinyl-4-(3′-chloro-4′-methyl-phenyl)-piperazine (Hoechst S 688) were found to have a strong chemotherapeutic effect on mature infections of *Schistosoma mansoni* (Puerto Rican) in white mice and monkeys. Some of these animals were completely cured of their infection. The dosage level necessary to bring about clearance of monkeys of all their schistosome parasites approached toxic levels. There was no evidence of curative effect on a *S. japonicum* (Formosan) infection in a dog which was given a low dosage of the chemical. When S 688 was given to mice up to 72 hours after exposure to *S. mansoni*, it prevented many of the larval schistosomes from developing. This was not observed in monkeys. There was a suggestion that the chemical had prophylactic activity in preventing some of the *S. japonicum* infection from developing in dogs treated for the three days after the exposure day. The present results are in agreement with those of de Meillon, England and Lammler (1956), and Lammler (1958) who discovered the curative and prophylactic activity of the compound against South African and Liberian *S. mansoni* in rodents and monkeys.

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AN ENRICHED, EGG EXTRACT MEDIUM FOR RECOVERING VIABLE *ENTAMOEBA HISTOLYTICA* TROPHOZOITES FROM FECAL SUSPENSIONS KEPT AT ROOM TEMPERATURE FOR FOUR DAYS

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PVA[3] and MIF fixatives[5] have been shown to be satisfactory for shipping fixed trophozoites of intestinal amebae, but no medium has been reported which makes possible the shipment of viable trophozoites in suspensions of the original fecal material. Such a medium would have two main uses. First, it would be convenient for obtaining new amebic strains from field surveys, since limitations of time, equipment and personnel often make cultivation procedures impractical in the field. Second, it would enable a central diagnostic laboratory to make identifications on the basis of living, motile amebae as well as on fixed, stained ones.

Earlier studies in this laboratory had shown that by using antibiotics and certain technical procedures trophozoites could be kept alive for up to four days at room temperature in suspensions of feces.[4] However, results with the culture medium employed at that time had been erratic. In the course of maintaining stock cultures of *Entamoeba histolytica* in this laboratory on egg extract medium,[1] attempts were made to improve growth by adding thioglycollate, horse serum, dextrose and Trypticase to the medium. These factors were chosen on the basis of work reported by Shaffer,[6] and Becker and Geiman.[2] All stock cultures responded so well to these additives that we were led to reconsider the earlier project on the development of a shipping medium for trophozoites of *E. histolytica* in fecal suspensions. The present paper then, describes the use of an enriched medium as a shipping solution primarily for trophozoites in feces.

MATERIALS AND METHODS

A large number of trials with varying combinations of the above-mentioned substances showed that best growth was obtained in medium prepared as follows: To 5 parts of Balamuth's egg extract medium without liver was added 1 part of the following solution: 100 ml Locke's solution containing 110 mg dextrose, 2 mg Trypticase (BBL), and 50 mg sodium thioglycollate. The enriched medium was dispensed in 6 ml volumes in 15 x 125 mm test tubes.

Three strains of *E. histolytica* (K-9, Huff and F-22 (a) and (b)) and two strains of *E. hartmanni* (335 and M.A.) were used in these studies.* All stocks were maintained with mixed unidentified bacteria on egg extract medium.

The survival of *E. histolytica* trophozoites in fecal suspension at 25°C for up to 4 days was investigated. One-half of an ml of a heavy suspension of human feces in normal saline was added to each of several tubes of enriched medium containing, in addition, 1000 units/ml of penicillin sodium and 2 or 3 mg/ml of streptomycin sulfate. The amount of fecal suspension added was estimated to parallel or slightly exceed the amount of feces used when culturing amebae from stools. Seven different fecal specimens, obtained from four individuals, were used in these experiments. Careful examination had not demonstrated any natural infections with amebae in these individuals. Amebae from stock cultures were pooled and hemocytometer counts made. Each tube was then inoculated with a known number of trophozoites suspended in 0.5 ml of enriched medium. All tubes were layered with melted paraffin. Control tubes were incubated at 37°C on the day of inoculation. Experimental tubes were stored at 25°C for from 1 to 4 days and then incubated at 37°C. Because of the regularity of survival of amebae after 1 or 2 days at room temperature, only 1 cul-

* K-9—Isolated in Korea in 1952 by members of the Subcommittee on Amebiasis of the Commission on Enteric Infections, Armed Forces Epidemiological Board; Huff and F-22(a)—Obtained from Dr. P. C. Beaver at Tulane University, New Orleans; F-22(b)—Obtained from Dr. W. W. Frye, Tulane University, New Orleans; 335—Isolated in Georgia in 1952 by Norman and Brooke; M.A.—Isolated by this laboratory in March, 1958.

ture tube was used for each amebic strain tested for these time periods. For the 3 and 4 day storage periods, 2 or 3 tubes were used for each strain. Before transfer to 37°C, the supernatant in each tube was replaced with fresh, enriched medium without antibiotics. Cultures were examined daily. They were not considered positive, i.e., capable of being subcultured, unless growth was 2+ or better (3–10 amebae/low-power field (LPF).

In the second phase of the study, two brief field trials were made. In one case, 2 culture tubes for each of 3 strains were prepared as described above, except that screw-capped tubes were employed. These were hand-carried by air to Texas on the day of preparation and sent back to the laboratory by air mail, arriving approximately 48 hours later. Upon receipt, the supernatant fluid was replaced with fresh medium and the tubes were incubated at 37°C.

In the second instance, original isolations were made in the field. Twenty-four purged stools were collected at a mental institution in Mississippi during the month of August. Three of these showed *E. histolytica* trophozoites. Portions of each were inoculated into duplicate sets of 3 tubes each of enriched medium plus antibiotics. One set of cultures was mailed to the laboratory on the day of inoculation; the other was incubated immediately at 37°C for one day. They were then carried by car to Chamblee, Georgia, being subjected to the varying temperatures of Mississippi and Georgia summer weather for about 43 hours. On arrival at the laboratory, the same procedures were followed as for the laboratory experimental cultures.

RESULTS

All 5 strains of amebae reached 2+ or better growth within 24 hours when incubated at 37°C after 1-day storage at room temperature. Cultures stored for 2 days grew out within 24 to 72 hours. About two-thirds of cultures stored for 3 days grew out within 3 to 5 days. Cultures stored for 4 days generally required somewhat longer to grow out; over half of them took 4 to 6 days, with a minimum of 3 days and a maximum of 10. Different fecal suspensions had markedly different effects on the growth of amebae. In some cases, cultures stored for 3 or 4 days grew out within 4 days when incubated. In other instances only rare active amebae were seen for 5 or 6 days, after which many cultures became 2+ or better in an additional 1 to 2 days.

The size and condition of the inoculum influenced the number of positive results. Growth was obtained most consistently with inocula of between 20,000 and 30,000 trophozoites per tube. In one experiment where 4 tubes received inocula of 10,000 amebae each, none grew out. In another experiment 4 tubes with inocula of 15,000 amebae reached 2+ growth after 3 days at room temperature, but only 1 of 4 grew out after the 4-day storage period.

In the laboratory phase of the investigation, 78 per cent of cultures stored for 3 days, and 72 per cent of those stored for 4 days, grew out into good cultures when incubated at 37°C. Approximately 85% of cultures called 1+ at some stage of these experiments became 2+ or better subsequently. Thus a few trophozoites were seen transiently in some tubes which never attained 2+ growth. A series of cultures similar in all respects to the experimental ones, except that no fecal suspension was added, generally required 24 to 48 hours longer to grow out than did cultures with added feces.

Of the laboratory-prepared cultures mailed from Texas, two of the three amebic strains grew out within 4 days after incubation. The third strain showed only rare active amebae at that time, but 3 days later it had reached 2+ growth. Both series of original isolations made in Mississippi grew out within 3 to 9 days after receipt and incubation in the laboratory.

DISCUSSION

In the original work performed in this laboratory on recovering trophozoites, it was found that a critical difference existed between the method of transferring amebae sediments from one tube to another and the method of pouring off old medium and replacing it with fresh in the same tube. With the former procedure, amebae could not be recovered after two days at room temperature, whereas with the latter, recovery was possible in several cases up to four days. In the present work, the efficacy of the replacement method was confirmed and approximately 75% of laboratory cultures were recovered after 3 and 4 days. Simitch *et al.*[7] have also emphasized the importance of this simple technique.

The different results obtained with different fecal samples in the laboratory experiments were

not surprising in view of probable differences in bacterial floras. It is more difficult to estimate the importance of this variation in attempted isolations from infected individuals. It is possible that amebae will survive more readily in bacterial suspensions derived from their own "normal" associates. On the other hand, the heavy antibiotic dosages used in the cultures might so alter the flora that the bacterial associates would not retain their original significance.

The possible role of cysts in the survival experiments reported here needs comment. In the stock cultures utilized in the laboratory experiments, cysts have not been seen in the Huff or 335 cultures either on direct examination or in stained slides made from time to time over a period of months. Rare cysts have been seen occasionally in the other strains. Cysts were never seen in cultures under observation following storage at room temperature, and the possibility that survival was due to rare cysts seems remote.

In contrast to the laboratory experiments, cysts may play a part in survival of cultures collected in the field. In one of the 3 cultures isolated in this study in Mississippi only trophozoites were observed at the time the specimen was collected but, upon receipt of the mailed cultures, many cysts were seen. Apparently encystment had taken place en route. This fact does not detract from the value of the enriched medium whose main purpose is to facilitate shipment of fecal samples in which only trophozoites are seen on direct examination. It should be pointed out, however, that another of the Mississippi isolations was from an acute case of amebiasis. This specimen contained originally, and after shipment, only large trophozoites with ingested red blood cells. We have never found cysts in this type of bloody mucoid feces.

In general, between 20,000 and 30,000 trophozoites were needed in order to obtain successful cultures after 3 or 4 days storage. Because of the many uncontrollable variables involved in mailing specimens, we have not attempted to define precisely how many amebae per field would need to be seen in the original stool in order to obtain positive cultures after mailing. It is probably safe to estimate, however, that if there are fewer than 2 or 3 amebae/LPF in a cover-slip preparation, few positive cultures would be obtained.

Although we have used 2+ growth as the minimum requirement for a successful culture, it might be possible to obtain the necessary diagnostic information from a lower level of growth. This would result in an increase in the number of positives.

The control experiment in which amebae were inoculated into enriched medium plus antibiotics but without fecal suspensions appeared to confirm the dependence of *E. histolytica* on an adequate number of metabolizing bacteria for good growth. The concentrations of antibiotics used were high enough to inhibit almost completely the bacteria added with the amebic inoculum. They were not high enough to prevent a fair degree of bacterial growth in cultures containing fecal suspension.

In our original work with a less efficient medium, a field experiment resulted in 8 positives following 48 hours in the mail, compared to 10 following immediate cultivation. Results obtained with mailed specimens in the present experiments tend to confirm the earlier findings. Since most state laboratories can be reached within 48 hours by air mail from anywhere in the state, survival for this time period is sufficient to make this method of shipping stools of diagnostic value to the public health laboratory.

The broad outlines of the approach to this problem seem clear as a result of these experiments. As the opportunity presents itself, we hope to undertake additional field studies in order that details of handling and shipping can be worked out, and improvements sought to shorten the time interval before cultures become positive.

SUMMARY

1. Balamuth's egg extract medium without liver, enriched with dextrose, Trypticase and sodium thioglycollate has been found to be superior to the original medium in ability to support *Entamoeba histolytica* in cultures with mixed bacteria.

2. Trophozoites of three strains of *E. histolytica* and of two strains of *E. hartmanni* were inoculated into the enriched medium plus antibiotics, a fecal suspension was added, and the tubes were stored at room temperature for up to 4 days. Upon incubation at 37°C, viable amebae were recovered from all tubes stored for 1 and 2 days, and from approximately 75% of those stored for 3 and 4 days.

3. In 2 field trials, 3 strains of laboratory-

prepared cultures containing fecal suspensions, and three original isolations of amebae from purged stools, were mailed to the laboratory. In all cases positive cultures were obtained upon cultivation of the tubes at 37°C, after the tubes had been in transit for 2 days.

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IN VITRO STUDIES ON THE SITES OF JAPANESE ENCEPHALITIS VIRUS MULTIPLICATION IN THE HERON, AN IMPORTANT NATURAL HOST IN JAPAN

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Studies at the US Army 406th Medical General Laboratory during 1952–1956 established that birds of the family *Ardeidae* (herons and egrets) are ecologically important avian hosts of Japanese encephalitis (JE) virus in Japan.[1] Following the bite of an infected vector mosquito, *Culex tritaeniorhynchus*, these birds become inapparently infected in nature and develop viremia and antibodies.[1, 2] Viremia usually appears within 1–3 days after inoculation of virus, lasts 3 or 4 days, and occurs at low titers, $10^{0-2.5}$ mouse LD_{50}/0.03 ml of blood.[2, 3] Because it is difficult during viremia to distinguish between virus produced locally in a tissue and virus from blood circulating through the tissue, one cannot easily identify the tissue sites of virus multiplication in birds by directly testing biopsied tissues for virus. Thus an approach to definition of the avian tissue trophisms of JE virus was sought in experiments with tissues cultured in vitro. The purposes of these studies were to determine which heron and egret tissues were capable of supporting growth of JE virus in vitro and to look for virus in tissues cultivated from herons long after viremia.

MATERIALS AND METHODS

Tissue cultures. Tissues from 10–12 month-old Black-crowned Night Herons or from heron embryos were cultivated as suspended tissue fragments in mixtures of rabbit serum and maintenance solution[4] modified to exclude the purines and pyrimidines. Herons and heron embryos were obtained during the summers of 1955 and 1956 respectively from the Shinhama Imperial Duck Refuge near Tokyo, Japan. Since heron eggs were difficult to identify by species, "heron embryo" is used to include embryos from Black-crowned Night Herons, *Nycticorax nycticorax*, Little Egrets, *Egretta garzetta*, and Plumed Egrets, *Egretta intermedia*. After mincing each tissue, approximately 10–20 fragments (1 mm diameter) were transferred to a 16 x 125 mm screw-capped test tube. Medium, composed of rabbit serum (shown by neutralization test in mice[2] to have a neutralization index less than 10), and maintenance solution, was added in 1 or 2 ml volume to each tube. Rabbit serum in different experiments ranged from 5 to 20% final concentration; penicillin and streptomycin were routinely employed in final concentrations of 500 units/ml. Cultures were incubated at 35–37°C.

Viruses. Ten strains of JE virus were inoculated into tissue cultures at selected dilutions of infected mouse brain containing between $10^{2.9}$ and $10^{4.7}$ weanling mouse LD_{50} of virus in different experiments. One strain, heron 281801, was isolated in mice from blood of a heron obtained at Shinhama in 1955, and was employed in third mouse passage. The sources and mouse passage levels of the other nine strains are given in Table 2. The diluent for preparation of mouse brain suspensions was either 5% dextrose in water or 10% rabbit serum in normal saline. Virus harvests were cultural fluids and tissues (from triplicate test tube cultures) triturated together in a Ten Broeck grinder and were stored in sealed glass ampules at −70°C on CO_2 ice.

Quantitation of virus harvested from tissue cultures. Virus was titrated by inoculating decimal dilutions intracranially into groups of 5 or 6 3–4 week old weanling mice, 0.03 ml/mouse. After mice had been observed for 14 days, LD_{50} were calculated by the method of Reed and Muench.[5]

RESULTS

Two experimental approaches were carried out in study of the trophism of JE virus in heron tissues. One was to propagate virus in tissue cultures from heron embryos and the other was an attempt to locate virus in tissues of previously infected herons that were no longer

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TABLE 1

Survival of JE virus, heron strain 281801, in the presence of degenerating tissue in cultures at 36°C

Tissues from 10–12 month-old herons*	Quantities of virus harvested from cultures at indicated times after inoculation				
	Min. 15	Days			
		1	3 or 4	7	10
	neg. log weanling mouse LD_{50}/0.03 ml IC				
Kidney, gonad, intestine, pancreas	1.5	<0	<0	<0	<0
	1.6	<0	<0	<0	<0
Stomach & esophagus	1.4	1.0	<0	<0	<0
Cerebrum, cerebellum & pons	1.8	1.3	<0	<0	<0
	1.8	1.0	<0	<0	<0

* Tissue fragments were suspended in a mixture of rabbit serum, 1 part, and maintenance solution, 19 parts.

viremic. Two additional experiments were done as preliminaries to the tissue culture studies; in one, the instability of JE virus in cultures without viable cells was determined, and in the other, virus strains from different sources were surveyed for their capacities to grow in heron tissues.

Instability of JE virus in tissue-free medium and in cultures of degenerating tissues. JE virus is known to be labile at 35–37° C in tissue cultures as commonly now employed.* In the absence of viable cells to support growth, Banta[7] reported complete disappearance of nearly 10^3 weanling mouse LD_{50} of the Nakayama strain of virus in 48 hours in a chicken serum-tryptose phosphate broth-maintenance solution mixture[8] at 36°C; moreover, as much as $10^{7.5}$ weanling mouse LD_{50} of the same strain becomes noninfectious in three days in a mixture of horse serum, 100 parts; yeast extract, 1 part; Hanks' balanced salt solution, 899 parts.[9] Virus has also been shown to deteriorate from titers of 10^7 hamster kidney TCD_{50}/0.1 ml to undetectable levels within 4 days after total destruction of cells in culture.[10]

To evaluate the possibility that JE virus might become stabilized in cultures of heron tissues without multiplying, the following experiments were performed. On 5 occasions $10^{3.2}$ or $10^{3.4}$ weanling mouse LD_{50} of virus were inoculated into suspended tissue fragment cultures of adult heron tissues, the cells of which usually degenerate and fail to reduce the pH of medium within a day or so of preparation.† Yet even in the presence of degenerating tissues, virus uniformly became undetectable within 3 or 4 days at 36°C (Table 1). Thus it was concluded that the presence of JE virus beyond the 3rd or 4th days of cultivation could be taken as presumptive evidence of virus reproduction, and that maintenance of initial virus titer or an increase in titer during 3 or more days of incubation, was definite evidence of virus multiplication.

The next step was to survey the reproductive capacities of nine virus strains in heron embryonic tissue cultures to learn whether there were differences among strains isolated from birds versus those recovered from mosquitoes or man.

Comparison of JE virus strains from different sources for capacity to grow in tissue cultures of minced heron embryos. Nine strains of JE virus originally isolated from heron blood, mosquitoes, or human brain or spinal fluid were inoculated in quantities of $10^{2.9-4.1}$ weanling mouse LD_{50} into suspended tissue fragment cultures prepared from whole heron embryos. Embryonic tissues were used because of their uniform ability to provide viable cells for 7–10 days in the type of cultures employed.

Each strain of virus multiplied in cultures of heron embryonic tissues (Table 2). Either the virus titer increased or remained constant for 7 days, and although low, it was as high as virus titers found in heron blood during viremia.[2, 3] Since there were no significant differences in

* Perhaps the instability of JE virus in cultures is related in part to the only slightly alkaline, neutral or even acid pH's that occur. However, although the stability of JE virus is enhanced by alkalinity to a pH of 8.5 (6), such alkalinity cannot be used in tissue cultures because it is incompatible with cell viability.

† Rather than calculate initial virus titers immediately after inoculation of cultures from quantities of virus inoculated, it was chosen in these experiments to measure virus titers 15 minutes after inoculation. The measured values, however, were essentially equal to the calculated titers. For example, when $10^{3.2}$ LD_{50} were inoculated into 2 ml of medium in kidney, gonad, intestine and pancreas cultures (Table 1), the calculated titer (*i.e.*, virus concentration per 0.03 ml inoculum per mouse) was $10^{-3.2}/10^{-1.8}$ $(10^{-1.8} = 0.03/2) = 10^{-1.4}$; the measured titer was $10^{-1.5}$ (Table 1).

TABLE 2

*Multiplication of JE virus strains from different sources in tissue cultures of minced whole heron embryos**

JE virus strain			Quantities of virus harvested from cultures at indicated times after inoculation			
Original source	Designation	Mouse passage used as inoculum	Minutes 15	Days 1	4	7
			Neg. log weanling mouse LD_{50}/0.03 ml IC			
Heron blood						
1955	281777	3	1.6	2.3	2.3	1.8
1955	281776	3	1.6	1.5	2.3	1.6
Mosquito (*C. tritaeniorhynchus*)						
1955	M5/572	2	1.8	1.5	2.5	1.5
1955	M5/560b	2	2.5	1.5	2.6	1.6
1955	M5/596	2	1.6	>1.4	2.3	1.8
1951	M1/311	11	n.t.†	1.6	3.1	2.1
Human brain						
1955	55-1735	2	1.6	0.5	2.4	1.8
1955	55-1456	2	1.6	1.5	2.5	n.t.
Human spinal fluid, 1935	Nakayama	58–68	1.8	1.6	1.8	1.8

* Embryos were 20 days of age for cultivation of virus strain M1/311, and 9–12 days of age for the other viruses. The medium was a mixture of rabbit serum, 2 parts, and maintenance solution, 8 parts.

† n.t. = not tested.

degrees of reproduction among these nine virus strains, only three were used in the next experiment in an attempt to propagate JE virus serially in selected tissues from embryos.

Multiplication of JE virus in cultures of selected pools of tissues or single tissues from 12–20 day heron embryos. In one set of experiments an attempt was made to propagate the Nakayama strain of JE virus, a mosquito strain, M5/572, and a heron strain, 281801, serially in cultures of heron embryonic tissues. The fluid medium in each tube culture was 1 ml of a mixture of rabbit serum, 1 part, and maintenance solution, 19 parts. At 5-day intervals, fluid and tissues from 3 test tube cultures were pooled, the tissues triturated in the fluid, an aliquot (0.1 ml) transferred to each of three new tissue cultures (prepared from another heron embryo) and another aliquot frozen at −70°C for eventual quantitation of virus in mice. Each strain of virus propagated through three or four serial passages in cultures of pooled heart and thigh muscle, and of lung and kidney tissues (Table 3). At the end of the fourth passage after a cumulative dilution of 10^4, virus was still present at titers of $10^{0.7\text{-}2.2}$ mouse LD_{50}/0.03 ml. Similarly the Nakayama strain and the mosquito strain, M5/572, propagated in liver and spleen tissues. On the other hand, the evidence for virus propagation in cultures of stomach and intestinal tissues was inconclusive, and virus could not be propagated serially at 5-day intervals in brain tissue. Perhaps the failure of brain tissue to propagate virus was related to poor maintenance of brain cells in suspended tissue fragment cultures for periods as long as 5 days.

In another set of experiments, the virus titer in cultures was determined at intervals after inoculation. Two virus strains, heron 281801, and mosquito M1/311, either increased in quantity or maintained approximately the same titers over 7-day periods in cultures of heart and thigh muscle, lung and kidney, and liver and spleen (Table 4).

TABLE 3

Serial propagation of JE virus in tissue cultures from 12–20 day heron embryos

Heron embryonic tissues	Virus passage number	Total days in culture	Cum. log dilution of first passage 1/8th day harvest	Quantities of virus harvested		
				Nakayama strain	Mosquito strain M5/572	Heron strain 281801
				neg. log weanling mouse LD_{50}/0.03 ml IC		
Heart & thigh muscle	1	⅛th	0	2.4	3.2	2.5
	1	5	1	>0	>0	>0
	2	10	2	1.8	1.9	1.5
	3	15	3	1.5	<0	1.2
	4	20	4	0.8	0.7	1.5
Lung & kidney	1	⅛th	0	3.0	2.5	2.5
	1	5	1	>0	>0	>0
	2	10	2	2.5	2.2	3.0
	3	15	3	1.6	<0	1.5
	4	20	4	2.2	1.5	n.d.*
Liver & spleen	1	⅛th	0	2.5	2.3	1.8
	1	5	1	>0	>0	>0
	2	10	2	1.6	1.5	<0
	3	15	3	2.0	1.5	<0
	4	20	4	1.5	0.5	<0
Stomach & intestine	1	⅛th	0	2.4	2.6	2.7
	1	5	1	>0	>0	>0
	2	10	2	0.4	1.6	1.5
	3	15	3	0.5	<0	0.2
	4	20	4	n.d.	<0	n.d.
Brain	1	⅛th	0	2.8	1.8	2.5
	1	5	1	>0	>0	>0
	2	10	2	<0	0.4	<0
	3	15	3	<0	<0	<0
	4	20	4	n.d.	<0	<0

* n.d. = not done.

To show that heron embryonic tissues were not unique in their ability to support virus multiplication, a number of attempts were made to make adult heron tissues survive for 7–10 days in suspended tissue fragment cultures. In one instance, cultures of a pool of heart, thigh muscle and lung from a juvenile (10–12 month old) heron maintained detectable virus over a 10-day period; the 15-minute and 1st, 3rd, 7th and 10th day titers were $10^{1.5}$, 10^{0}, $10^{0.3}$, $10^{1.5}$ and $10^{0.6}$ weanling mouse LD_{50}/0.03 ml. Thus adult tissues apparently were similar to embryonic tissues in their trophism for JE virus.

In the next experiment, an attempt was made to learn more precisely which heron tissues in the previously employed pools supported virus growth. Using procedures identical with those of the previous experiments, it was found that heart, thigh muscle, lung, liver and spleen support virus multiplication in vitro (Table 5). Also of interest in this experiment was evidence of viral reproduction in stomach and intestinal tissues and in brain, whereas in the previous experiment (Table 3) repeated harvests and passages of virus from cultures of these tissues at 5-day intervals eventually resulted in disappearance of virus. This observation suggests that if virus in the previous experiment had been passed serially at intervals of less than 5 days (*i.e.*, when viable cells were still present

TABLE 4

Multiplication of JE virus in tissue cultures from 14–20 day heron embryos

Heron embryonic tissues	Virus strain	Quantities of virus harvested from cultures at indicated times after inoculation			
		Minutes 15	Days		
			1	3 or 4	7
		neg. log weanling mouse LD_{50}/0.03 ml IC			
Heart & thigh muscle	Heron 281801	1.7	1.2	1.2	1.5
	Mosq M1/311	1.5	1.5	1.8	1.8
Lung & kidney	Heron 281801	2.5	0.6	1.7	2.2
	Mosq M1/311	1.6	1.5	1.3	2.5
Liver & spleen	Mosq M1/311	1.6	1.8	2.1	2.3

TABLE 5

Multiplication of JE virus in cultures of single tissues from 14–20 day heron embryos

Heron embryonic tissues	Virus strain	Quantities of virus harvested from cultures at indicated times after inoculation			
		Minutes 15	Days		
			1	3 or 4	7
		neg. log weanling mouse LD_{50}/0.03 ml IC			
Heart	Heron 281801	1.8	1.2	1.6	1.2
Thigh muscle	Heron 281801	1.5	0.5	1.5	1.4
Lung	Heron 281801	2.7	1.0	1.6	1.5
Liver	Heron 281801	2.7	1.5	2.0	1.3
Spleen	Heron 281801	1.8	1.6	1.9	1.6
Stomach & intestine	Mosq M1/311	1.6	1.5	1.8	1.8
Brain	Heron 281801	2.0	0.8	1.5	1.8
	Mosq M1/311	1.5	1.6	2.3	1.8

in the cultures), virus propagation might have been demonstrated in stomach, intestinal and brain tissues.

Finally an attempt was made to find virus in tissues of herons after the period of viremia, as evidence of virus multiplication in the tissue. Since direct assay of biopsied tissues seemed inadvisable because of probable neutralization of virus by antibody upon trituration of the tissue, tissues were first cultivated in vitro before assay to allow time for metabolism and destruction of antibody and/or for growth of virus to detectable levels. This approach has been successfully employed for isolation of adenoviruses and rubeola virus from human tissues,[11-13] in finding *Rickettsia prowazeki* in tissues from patients with Brill's disease,[14] and in detection of poliovirus in kidneys of previously infected monkeys.[15]

Attempt to locate virus in tissues of previously infected herons. Five pools of tissues from each of 8 juvenile herons were cultivated 9–10 months after infection as suspended tissue fragment cultures in a mixture of rabbit serum, 1 part, and maintenance solution, 19 parts. The evidence for previous infection of these birds was as follows: 2 birds had demonstrable natural viremia on 29 July 1955, another had viremia after artificial inoculation of JE virus, strain M1/311, on 23 July 1955, and all birds had HI antibody upon test in August 1955. By April 1956 at the time of this experiment, 4 birds had no detectable neutralizing antibody in unheated plasma whereas the other 4 had antibody (a neutralization index > 10). The tissues were a) liver, spleen and femoral marrow, b) lung, heart and skeletal muscle, c) small intestine, kidney, gonad and pancreas, d) brain, and e) stomach and lower 1 cm of esophagus including the nematodes present. After 4–6 days of incubation at 36°C and again after 9 or 10 days, the contents of 3 cultures from each tissue pool were combined, triturated, and inoculated intracranially, 0.01 ml and subcutaneously, 0.03 ml, into 1–4 day old albino mice from two litters. For the tissues from one heron, weanling mice were also inoculated intracranially.

In contrast to the success of this method for isolating the viruses and rickettsia mentioned above, it failed in these 8 experiments to reveal JE virus in tissues of previously infected herons, since no deaths or illnesses of mice occurred that could be attributed to JE virus or another infectious agent.

SUMMARY

Japanese encephalitis virus was found to grow in vitro in a variety of tissues from the heron, an important avian host of virus in Japan. Cultures of heart, thigh muscle, lung, liver, spleen, stomach and intestine, and brain produced titers of virus similar to those which occur

in blood of naturally infected herons. These findings suggest that viremia in herons results from virus proliferation in many tissues, and that if, like western equine encephalomyelitis virus[16] JE virus remains dormant in birds, it might do so in a number of tissues rather than in a single tissue. However, no evidence was found of sequestered virus in 8 herons whose tissues were cultured first in vitro before inoculation of suckling mice.

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A COMPARATIVE STUDY OF THE SUBCUTANEOUS INOCULATION OF GERMFREE AND CONVENTIONAL GUINEA PIGS WITH TWO STRAINS OF *TRICHOMONAS VAGINALIS**

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Efforts have been made to establish *Trichomonas vaginalis* infections in the genital tract of germfree guinea pigs at ages varying from 3 weeks to 3–4 months. The rationale behind these attempts has been that, if such infections could be obtained, the organism and its behavior could be studied apart from whatever influences might be exerted by the flora accompanying this infection under normal conditions. Information on such matters as variation in strain virulence and the pathology produced by the organism *per se* could be obtained, and would, perhaps, help explain the wide variation in the clinical picture presented by cases of this infection. It is, of course, apparent from reviews[13, 6] on the attempts to infect animals with this parasite that the guinea pig has proved to be a rather unsuitable host. However, this was the only germfree animal available to us at the time. Also, increased susceptibility of the germfree guinea pig (over the conventional animal) to other parasites has already been demonstrated.[7]

Thus far, we have not established the infection in germfree guinea pigs by direct inoculation of *T. vaginalis* into the genital tract, although the concomitant use of hormones, a procedure which resulted in infections in albino rats[3, 4] has not as yet been attempted. However, during one of the trials, a few germfree guinea pigs were inoculated subcutaneously with the parasite. A rather severe lesion was obtained, and this prompted further study of the behavior of this parasite when injected under the skin of both germfree and conventional guinea pigs.

MATERIALS AND METHODS

The germfree guinea pigs were obtained by Caesarean section from pregnant conventional dams of the NIH general purpose colony, and maintained in Reyniers Germfree System units. The techniques for obtaining and rearing the animals, the diet employed and the methods of checking sterility have been reported in detail elsewhere.[9, 7] The conventional animals were drawn from the same colony at the age of 3–5 days. Some were maintained on the usual laboratory regimen of kale, carrots, and commercial chow. Others were reared on the same sterilized food given the germfree guinea pigs.

Two strains of *T. vaginalis* maintained in axenic culture according to the methods of Burch, Rees, and Reardon,[1] were used. One strain, R, was obtained originally from a mild human infection, and the other, strain C, from a patient with a severe, highly symptomatic case of vaginitis. Antibiotics were used to establish these strains in axenic culture. At the time of this study, the antibiotics had long since been dispensed with, and the parasites had been maintained in vitro for 2–3 years.

For the experiments the trichomonads were grown in 250 ml centrifuge bottles and harvested after 48 hours incubation at 35°C. The organisms were concentrated by centrifugation, the supernatant fluid decanted, and the sediment suspended in saline, usually about 3 ml per bottle. The suspension from each bottle was then transferred to a 5 ml sterile ampoule. All procedures were carried out under strict aseptic conditions to preclude extraneous contamination. The ampoules were sealed by flame and introduced into the germfree units via a germicide trap. The residues remaining in the centrifuge bottles were used for hemocytometer counts for an approximate calculation of the number of organisms per ml.

Both germfree and conventional guinea pigs were injected from the same lot of inoculum in a given experiment. The usual procedure was to inject approximately 1 ml of inoculum subcutaneously in the neck scruff area. In the experiments reported here, approximately 5×10^7 organisms were introduced with each injection,

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FIG. 1. Grade 0, R strain of *Trichomonas vaginalis* inoculated subcutaneously into conventional guinea pig. Note characteristic layers of fat, no evidence of the inoculation.

FIG. 2. Grade 1, C strain of *Trichomonas vaginalis* inoculated subcutaneously into conventional guinea pig. Arrows point to small accumulation of pus-like material. A few viable organisms were present.

and both C and R strains were used in each experiment.

The animals were 3 weeks old when inoculated. They were sacrificed 2 weeks later, and the injected area was examined grossly for the presence of lesions. Material from the site was examined microscopically for the presence of trichomonads. Each time the germfree animals on an experiment were removed from the unit for necropsy, a sterility check was made to assure that no other organism could be detected.

RESULTS

The inoculations produced a variety of results, depending upon the strain of trichomonad used, and upon whether the animal was germfree or was a conventional one. Findings ranged from no gross evidence of the inoculation to lesions, abscesses, and cysts up to 2 inches in diameter, some of which were filled with gas. Numerous active trichomonads were observed in microscopic examination of the pus-like material taken from lesions in many of the germfree animals.

In an effort to make a quantitative comparison of the experimental groupings, a grade of 0, 1, 2, or 3 was given to the finding in each animal. In an animal graded 0, no gross evidence of the inoculation was seen in the subcutaneous area around the injection site. (See Figure 1.) A small, hard, fibrous cyst, or an accumulation of pus-like material, a few millimeters in diameter, was characteristic of a grade 1 infection. (See Figure 2.) Occasionally a few viable trichomonads could be found. Such infections did not appear to be active, and could be considered as retrograde. Grades 2 and 3 were given to progressively larger lesions several millimeters to a few centimeters in size. As shown in Figure 3, these were usually palpable externally as a nodule or blister. Internally, tissue erosion, numerous active organisms, considerable pus or caseous material, and often relatively large amounts of gas were present. (See Figures 4 and 5.) The cutaneous layers around the inoculation site were often raised in a turgid "blister," and from one animal as much as 15 ml of gas were removed under an oil seal by

FIG. 3. Grade 2, R strain of *Trichomonas vaginalis* inoculated subcutaneously into germfree guinea pig. External view showing easily palpable nodules.

FIG. 4. Grade 2, R strain of *Trichomonas vaginalis* inoculated subcutaneously into germfree guinea pig. Note large accumulations of caseous material throughout.

needle and syringe. (See Figure 6.) When this lesion was opened, a frothy mixture of pus and trichomonads, together with evidence of tissue erosion, was observed (Figure 7).

In Table 1 are presented the combined results of three experiments involving 23 germfree and 27 conventional guinea pigs, all of which received approximately 5×10^7 trichomonads each. These results are typical of those obtained with other experiments in which the number of organisms inoculated or the age of animals may have differed slightly. The conventional animals on both the "germfree" type of diet and the stock guinea pig diet are grouped in the table inasmuch as a diet effect was not noted. Two facts are apparent from the Table: 1) Subcutaneous infection was established more easily in the germfree than in the conventional guinea pigs. 2) The C strain was more virulent than the R strain.

All but one of the germfree animals inoculated with strain C had lesions, in most cases of grade 3 intensity. In contrast, and with but one exception, the conventional animals showed either no evidence of the inoculation or at most only a small residuum. In the case of the R strain of the trichomonad, there was some indication that the parasites survived a little better in the germfree animals than in the conventional ones. No evidence of the inoculation was found in 11 of 13 of the latter, whereas most of the germfree animals showed a little residuum and two had a grade 2 infection. However, in general, the strain did not survive as well, or exhibit the same pathogenicity as the C strain. If one uses the average grade per animal as a rough rule for comparison, the difference between the strains is readily apparent, particularly with respect to their behavior in the germfree animals.

DISCUSSION

On the basis of the results with the C strain of *T. vaginalis*, the germfree guinea pigs were more susceptible to the parasite than were the conventional animals. In view of the controls attempted, the main apparent difference between the two groups was the fact that one was reared free of demonstrable viable organisms, whereas

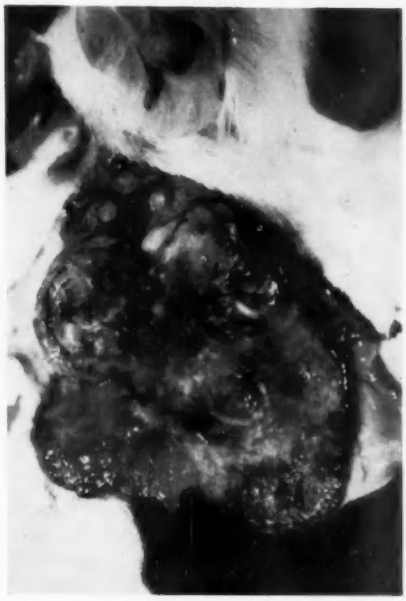

FIG. 5. Grade 3, C strain of *Trichomonas vaginalis* inoculated subcutaneously into germfree guinea pig. Note frothy evidence of gas production at A, and pus-like accumulation at B. Numerous active trichomonads were present.

FIG. 6. Grade 3, C strain of *Trichomonas vaginalis* inoculated subcutaneously into germfree guinea pig. External view showing cutaneous area raised in a large blister.

the other was in constant association with a varied flora. The subcutaneous area could be expected to be about the same, in a broad physiologic sense, in both animals, and the nutritional and environmental essentials for the growth of the parasite might be expected to exist in one if they were present in the other. Thus, it would appear that the failure of the parasite to prosper in the conventional animals was due not to the lack of such essentials but rather to some tissue response or other defense mechanisms not present, or underdeveloped, in the germfree animals. If one pursued this line of reasoning further, he could conclude that the constant presence of a flora stimulated the development and maintenance of certain, seemingly nonspecific, aspects of the conventional animals' defense mechanism.

It is possible that the failure of the germfree animals to have obtained mother's milk, especially the colostrum, during the first few days of life, as did the conventional animals, may have been a factor. However, in studies in which these these animals were inoculated with parasitic nematodes,[7] this difference did not prove to be decisive in the different infection results obtained between germfree and conventional guinea pigs.

Of interest was the apparent difference in virulence between the C and R strains of the parasite. Similar differences have been noted by others[5, 2] who inoculated white mice with various strains of *T. vaginalis*, subcutaneously or intraperitoneally, and compared lesion size or mortality. When the same strains of *T. vaginalis* as we used were inoculated intraperitoneally into white mice, a strain difference was noted; the C strain was quite lethal and the R strain was not.[12] The various findings all suggest that the different clinical pictures observed among human cases of trichomoniasis can be due, to a considerable extent, to differences among the strains of parasites involved. However, in some tests,[13] there has not always been a clearcut correlation between the symptomatology in cases from which the strains were derived and their lethality for mice.

The poor development of the R strain in the subcutaneous area of both groups of animals may

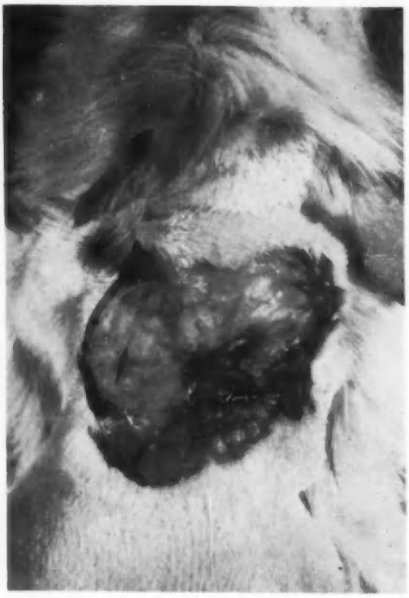

FIG. 7. Same animal as in Figure 6, with skin of blister laid back. Note evidence of gas production. Numerous organisms and cellular elements present.

TABLE 1

Results of subcutaneous inoculation of germfree and conventional guinea pigs with two strains of Trichomonas vaginalis *into neck scruff area, 2 weeks post-inoculation*

Type of animal	Strain of trichomonad	Total number of animals	Number showing lesions at inoculation site graded* as follows:				Average grade per animal
			0	1	2	3	
Germfree	C	12	—	1	4	7	2.6
Conventional	C	14	6	7	1	—	0.6
Germfree	R	11	2	7	2	—	1.0
Conventional	R	13	11	2	—	—	0.2

* 0 = No evidence of the inoculation. 1 = *Internally*, small (1–4 mm) accumulation of yellowish, pus-like material, sometimes loose and sometimes enclosed in a thick hard fibrous capsule; a few live trichomonads may be present. 2 = *Externally*, 5–15 mm nodule palpable. *Internally*, cyst-like body containing considerable caseous material; some evidence of tissue damage; numerous active trichomonads. 3 = *Externally*, large nodule or cyst palpable, usually containing a large amount of gas. *Internally*, considerable fluid; some pus-like material; some tissue erosion apparent; many active trichomonads present.

have been a reflection of the unsuitability of this environment for the growth of the parasite as well as of the host's defensive mechanisms. Even in the germfree animal this strain did not do very well. Also, while the R strain may have produced small amounts of gas at some stage of the infection, at necropsy there was none of the large gas-filled blisters observed in animals inoculated with the C strain. It is to be wondered whether this was merely a reflection of poor growth and comparatively slower metabolic activity, whether the R strain is not a gas producer, or whether it does not produce the particular gas(es) that may have caused the blister in the animals given the C strain. In vitro, the R strain actually grows more rapidly than the C strain. While substrate and environmental factors are highly determinative in gas production,[10] it is also quite possible that there may be strain variation in this regard. It is interesting to speculate on whether gas production, particularly of a certain kind, is related to virulence in any way.

Examination by means of the mass spectrometer* of samples of the gas from the lesions on a few of the animals inoculated with the C strain revealed the presence of the variety of the gases that occur in the blood, in addition to a rather significantly high percentage of hydrogen, as much as 26% in one instance. While the data are yet too few to attempt to assess the significance of the various gas percentages, in view of the diffusion rate of hydrogen under such conditions it is apparent that the parasite was producing large quantities of this gas. In vitro gas production by *T. vaginalis* has been observed by others.[8, 11] In studies on the anaerobic metabolism of a strain designated 5-A, it was reported that hydrogen was among gases produced.[11] Gas evolution was studied in paired Barcroft-Warburg flasks, one with, and one without, alkali in the center well.

One final point is apparent from these studies.

* We wish to thank Dr. Arnold Pratt, Laboratory of Physiology, National Cancer Institute, for the gas analysis. The analysis was performed on a CEC MODEL, No. 21-102, 180° analytical instrument.

This is the fact that through the use of a germfree animal, in this case the guinea pig, characteristics of, and differences between, strains of an infectious organism could be demonstrated which were barely, if at all, detectable when the conventional counterpart was used.

SUMMARY

Several million organisms of two strains of *Trichomonas vaginalis* reared axenically were inoculated subcutaneously into the neck scruff area of germfree and conventional guinea pigs. One strain, C, was derived originally from a highly symptomatic case of trichomoniasis, and the other, strain R, from a mild case.

Large lesions containing many active organisms and, often, large quantities of gas were found 2 weeks post-inoculation in the germfree animals inoculated with the C strain. In the conventional animals, little or no evidence of the infection was the usual finding.

The R strain survived to some extent but only rarely caused a significant lesion in the germfree animals. In the conventional animals there was essentially no evidence of the inoculation.

The results indicated that the C strain of *T. vaginalis* was more pathogenic than the R strain, and that the germfree guinea pigs were more susceptible to tissue infection with this parasite than were the conventional animals. The possible significance of these findings is discussed.

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ISOLATION OF GERMISTON VIRUS, A HITHERTO UNKNOWN AGENT, FROM CULICINE MOSQUITOES, AND A REPORT OF INFECTION IN TWO LABORATORY WORKERS*

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This report describes the isolation of two virus strains from culicine mosquitoes collected in the course of an investigation of the etiology of fevers of unknown origin occurring at Germiston, an industrialized urban community located near Johannesburg, Union of South Africa. Immunological observations resulting from the study of this virus indicate that it is related to but sufficiently distinct from Bunyamwera virus to warrant a separate designation. Thus it is referred to as Germiston virus in reference to the community of the same name where field studies were conducted.

METHODS AND MATERIALS

Mosquitoes were collected individually in glass tubes 90 x 15 mm that were afterwards plugged with nonabsorbent cotton. All collections were made with man as bait. Between April 2–15, 1958, 11 catches were made along reed-beds at Germiston Lake (Figure 1). This was commonly done in the late afternoon, following which the mosquitoes were taken to the laboratory, identified and processed for attempted virus isolation the following morning by a previously described technique.[8]

Three types of serological test were employed in the course of these studies. Neutralization tests (NT) were done according to methods described by Smithburn.[16] Fifty per cent endpoints were determined by the method of Reed and Muench.[14] The techniques for the preparation of antigens and mouse hyperimmune serum and the methods for doing complement-fixation (CF) and hemagglutination-inhibition (HI) tests have been described.[2, 3, 6] In order to obtain homologous immune serum, selected nonimmune laboratory animals were inoculated. Germiston virus (AR 1050 strain) immune serum was obtained by pooling blood collected from 12 guinea-pigs 33 days after an intracerebral inoculation of $10^{2.7}$ intracerebral adult mouse LD_{50} of the homologous agent.

A *Cercopithecus aethiops pygerythrus* F. Cuvier monkey was inoculated intraperitoneally with $10^{8.8}$ intracerebral adult mouse LD_{50} of Bunyamwera virus. The sera used in neutralization tests were obtained from 1.5 to 2.5 years following this inoculation. However, for the hemagglutination-inhibition tests this monkey received a subcutaneous booster inoculation (4000 IC adult mouse LD_{50}) 39 months following the first. It was bled 13 days after this second inoculation with Bunyamwera virus and the serum used in HI tests.

Bunyamwera virus used to immunize adult mice was inactivated with beta-propiolactone following the general technique of lo Grippo and Hartman[11] but with modifications described by Clarke.[5]

Three strains of Bunyamwera virus were used in serological studies. One was the prototype strain[17] in its 44th mouse brain passage; the second, a strain isolated in Tongaland, Union of South Africa, designated AR 11 virus[6] in its 10th mouse brain passage; and the third (H 427) was isolated from a naturally infected human being[9] and used in its 14th mouse brain passage.

RESULTS

Virus isolation. One of the virus strains was isolated from a mixed pool designated AR 1050, which consisted of 5 *Culex* (*Culex*) *theileri* Theobald and 12 *Culex* (*Neoculex*) *rubinotus* Theobald. These were caught April 2, 1958 and processed for attempted virus isolation the following day. Of the two litters of newborn mice inocu-

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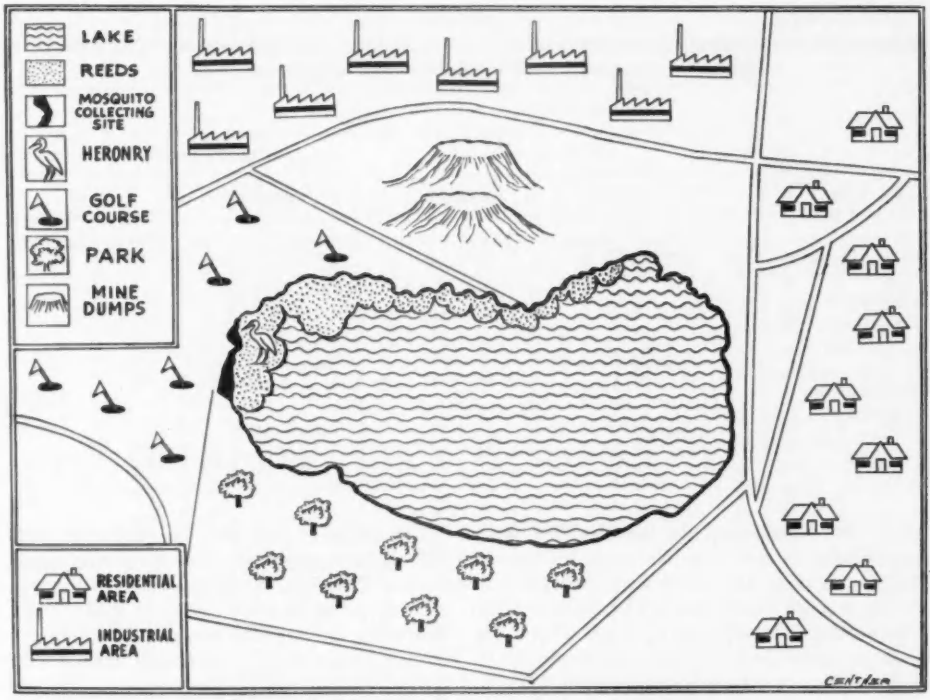

FIG. 1. Sketch map of Germiston Lake and environs

lated intracerebrally, one was unsatisfactory and the six mice in the other litter were found dead less than 48 hours postinoculation. Bacteriological cultures of the suspension prepared from the brains of these mice were sterile. An agent pathogenic to both newborn and adult mice was readily filtered through an asbestos filter pad (Seitz EK).

Adult mice inoculated with the mosquito suspension were sick after a 4-day incubation period. Specimens for further study were obtained from four mice and the two remaining mice died on the fifth and sixth days respectively.

Bacteriologically sterile suspensions of infected brain tissue were serially transmissible to mice of both age groups and the agent was subsequently referred to as AR 1050 virus strain.

None of the original mosquito suspension remained for attempted reisolation of the virus.

The second virus was isolated from a pool of 11 *Culex* (*Neoculex*) *rubinotus* Theobald designated AR 1057, collected April 10, 1958 and processed the next morning. Two litters of newborn mice and a group of six adult mice were inoculated intracerebrally with the mosquito suspension. The sequence of events in these mice following their inoculation was very similar to that occurring in the mice inoculated with AR 1050 mosquito suspension as described above. Potent lyophilized stocks of the agent were prepared and it was referred to as AR 1057 virus strain.

AR 1057 virus was successfully reisolated from the original mosquito suspension after storage at approximately −20°C for 3 days.

Immunological studies. In an initial cross-neutralization test, AR 1050 virus was tested against antisera for several African virus isolates having comparable incubation periods. Although no protective endpoint was determined in this test, Bunyamwera virus monkey immune serum neutralized over 1000 IC newborn mouse LD_{50} of AR 1050 virus. The virus was not neutralized by any of the other five potent antisera, namely West Nile, Rift Valley fever, Sindbis, Chikungunya and Semliki Forest.

Further cross-neutralization tests were done to

TABLE 1

Results of intracerebral adult mouse neutralization tests with 2 strains of Germiston virus and 2 strains of Bunyamwera virus and pre- and post-inoculation sera for each virus type

Serum	Virus type							
	Germiston				Bunyamwera			
	AR 1050		AR 1057		Bunyamwera		AR 11	
	Titer of virus, log	Logs virus neut.	Titer of virus, log	Logs virus neut.	Titer of virus, log	Logs virus neut.	Titer of virus, log	Logs virus neut.
Germiston G.P. group 57								
pre-inoc.	6.6		7.0		5.8		6.5	
post-inoc.	2.6	4.0	1.8	5.2	2.5	3.3	3.9	2.6
Bunyamwera M 230								
pre-inoc.	6.4		6.0		6.0		6.4	
post-inoc.	4.2	2.2	3.8	2.2	2.3	3.7	3.2	3.2

study this relationship. In each test pre- and postinoculation sera from the same laboratory hosts were used; the latter were prepared following a single inoculation of the specific virus. The results of these tests, in which adult mice were inoculated intracerebrally, are presented in Table 1. These tests demonstrate a close relationship between AR 1050 and AR 1057 virus strains on the one hand and Bunyamwera and AR 11 viruses on the other. Although not quantitative, the crosses are reciprocal.

Potent hemagglutinating antigens (HA) were prepared by acetone-ether treatment of sera from newborn mice sick after intracerebral inoculation of both AR 1050 and AR 1057 infected brain suspensions. Cross hemagglutination-inhibition tests were performed with: (1) these antigens, with a guinea-pig immune serum obtained after a single inoculation with AR 1050 virus, and (2) a Bunyamwera sucrose-acetone mouse brain extracted HA antigen and its homologous monkey immune serum obtained 13 days following a booster inoculation. These results are presented in Table 2. The close relationship of AR 1050 and AR 1057 viruses is again demonstrated. It is also evident that the acquisition of a potent immune response by the guinea-pigs (group 57) inoculated with AR 1050 virus resulted in no inhibition of Bunyamwera HA antigen. However, the Bunyamwera immune serum was strongly cross-reactive with both of the other virus strains.

The results of cross complement-fixation tests with antigens prepared by acetone-ether treatment of virus-infected mouse brains and hyperimmune mouse sera are shown in Table 3. AR 1050 strain of Germiston virus fixed complement in the presence of AR 1057 serum, but not with Bunyamwera mouse hyperimmune sera prepared from two strains of the latter agent. These results thus show the close relationship between AR 1050 and AR 1057 viruses, both considered to be

TABLE 2

*Cross hemagglutination-inhibition tests showing relationship of AR 1050 and AR 1057 virus strains and the inability of potent immune AR 1050 serum to significantly inhibit agglutination by Bunyamwera HA antigen**

Serum	Antigen		
	AR 1050	AR 1057	Bunyamwera
Germiston (AR 1050) G.P. group 57			
pre-inoc.	0		0
post-inoc.	320+	640+	0
Bunyamwera M 230			
pre-inoc.	0		0
post-inoc.	40	160	160

*HI titers expressed as reciprocal of highest dilution of serum that inhibited agglutination.

TABLE 3

Results of cross complement-fixation tests with Bunyamwera and Germiston (AR 1050) virus antigens and hyperimmune sera for each virus type

Antigen‡		Mouse hyperimmune serum*														
		Bunyamwera, 1 in					H 427, 1 in					AR 1057, 1 in				
		8	16	32	64	128	8	16	32	64	128	8	16	32	64	128
Bunyamwera																
1 in	4	4	4	4	4	2	4	2	0	0	0	0	0	0	0	0
	8	4	4	4	4	2	4	4	0	0	0	0	0	0	0	0
	16	4	4	4	4	3	4	4	2	1	0	0	0	0	0	0
	32	4	4	4	4	4	4	4	4	1	0	0	0	0	0	0
	64	4	4	4	4	3	4	4	3	1	0	0	0	0	0	0
	128	4	4	4	4	4	4	4	4	2	0	0	0	0	0	0
H 427																
1 in	4	4	4	4	4	4	4	4	0	0	0	0	0	0	0	0
	8	4	4	4	4	4	4	4	2	0	0	0	0	0	0	0
	16	4	4	4	4	4	4	4	3	1	0	0	0	0	0	0
	32	4	4	4	4	4	4	4	4	0	0	0	0	0	0	0
	64	4	4	4	4	4	4	4	4	1	0	0	0	0	0	0
	128	4	4	4	4	4	4	4	4	2	0	0	0	0	0	0
Germiston (AR 1050)																
1 in	4	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4
	8	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4
	16	0	0	0	0	0	0	0	0	0	0	4	4	4	4	1
	32	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	128	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Serum control negative.

‡ Normal mouse brain in a 1 in 4 dilution gave no evidence of fixation of complement.

strains of Germiston virus, and furthermore, confirm the relationship between the two strains of Bunyamwera virus. However, they clearly demonstrate the qualitative difference existing between Germiston and Bunyamwera viruses.

TABLE 4

Intracerebral challenge with Germiston (AR 1050) and Bunyamwera viruses of adult mice previously immunized with the latter agent

Treatment of mice	Challenge virus			
	Bunyamwera		Germiston	
	Titer of virus, logs	Logs virus protection	Titer of virus, logs	Logs virus protection
None (controls)	5.6		6.4	
Bunyamwera	3.2	2.4	5.8	0.6

The relationship of AR 1050 and Bunyamwera viruses was also studied by an immunization and challenge experiment. Adult mice were immunized by a series of two intraperitoneal inoculations of Bunyamwera virus killed with beta-propiolactone and a third injection, by the same route, of unattenuated virus. Fifteen days after the last injection, intracerebral challenge titrations of Bunyamwera and AR 1050 viruses were done in the immunized mice, and simultaneously in controls of the same age.

These results are presented in Table 4. They show that the immunizing injections evoked significant protection against Bunyamwera virus but did not produce resistance to Germiston virus.

Further immunological evidence that Germiston virus is not identical with Bunyamwera virus is presented below in the report of infection of two laboratory workers with the former agent.

TABLE 5

Results of serological studies with Germiston (AR 1050 strain) and Bunyamwera viruses or antigens against sera collected from case No. 1 (K) at various times prior to and following illness

Date patient's serum taken	Virus					
	Germiston			Bunyamwera		
	HI*	CF†	NT‡	HI	CF	NT
March 3, 1954			0			0
Feb. 18, 1955				0		
May 16, 1955	0	0	0	20	32	1.5
Aug. 10, 1956	0	0		0	0	
Sept. 30, 1957	0	0	0	0	0	3.0
April 11, 1958 (Onset of present illness)	0	0	0	0	0	3.5
April 18, 1958	40	8	1.8	40	16	4.0
April 24, 1958			2.5+	640+	64+	
March 3, 1959	1280	8	5.1	2650	32	4.8+

*HI titers expressed as reciprocals of highest dilution of serum that inhibited agglutination.

†CF titers expressed as the reciprocals of highest dilution of serum giving a 3-plus or better fixation.

‡NT titers expressed as the difference in logarithms of fifty per cent mortality end-points of the control titration and the titration in the presence of serum obtained on various dates.

Infections with Germiston virus in laboratory workers. Two infections occurred in persons intensively engaged in serological studies on new isolates. The more important findings relevant to these cases are as follows:

Case 1. K., white male aged 37, virologist, awoke from sound sleep at 04.05 hours April 11, 1958 with a chill and sensation of impending illness. His temperature was 98.2°F when first taken at 07.40. At 08.15 he felt suddenly warm and temperature was 101.4°F. Shortly after, excruciating bitemporal headache and lumbosacral pain were experienced. During the day the temperature was just over 102°F, but in the evening it fell to 99°F and with this decline the headache, which had persisted unabated during the day, subsided. During the next two days the symptoms gradually abated but he felt too ill to work. On April 14 he returned to work but felt weak and experienced some confusion and a tendency toward emotional lability. The facility with which numerous technical procedures could be done seemed to be impaired for a further 3 days.

Blood taken at 08.20 April 11 yielded no virus. The evidence of the etiology rests therefore on protection tests and other serological findings. An initial HI screening test with a seventh-day convalescent specimen showed antibodies for group B and Bunyamwera, but none for group A or Rift Valley fever viruses. It was then found that the acute and convalescent sera were both protective against a significant level of Bunyamwera virus, so recourse was taken to sera collected at various earlier times to study the response evoked by the current illness. The results presented in Table 5 are interpreted as indicating: 1. That the patient was infected with Bunyamwera virus in 1955 and probably had experienced only a partial antibody response by the time of bleeding on May 16, 1955. 2. That the CF and HI antibodies evoked by the 1955 infection disappeared entirely, but the neutralizing antibodies persisted. 3. That the illness in April 1958 evoked a response of HI and CF antibodies—probably an anamnestic response—to Bunyamwera virus. 4. That the 1958 illness evoked a response of CF, HI and neutralizing antibodies to AR 1050 antigen or virus. 5. That immunity to Bunyamwera virus did not protect the patient against clinically overt infection by AR 1050 virus. 6. That the HI and CF "conversions" for Bunyamwera between April 11 and April 18 would have been diagnostically misleading since the patient was immune to this agent long before that time.

Case 2. L., white female aged 21, technician, woke up on the morning of August 26, 1958 with aching legs and feeling very weak. She came to work but reported her illness at 10.30, at which time her temperature was 99.0°F. She did not feel capable of performing her normal laboratory duties and returned to her abode where she slept almost all day. There were no other complaints and by the following day she felt normal.

A blood specimen (H 131) was taken at 10.30 on August 26, 1958 and intracerebral inoculation with serum from this caused illness in infant and adult mice in 2 and 3 days respectively. The agent passed through a Seitz-EK filter pad and was serially transmissible. It was neutralized to the extent of more than 3.4 logs by AR 1050 guinea-pig immune serum. The donor's con-

TABLE 6

Results of three types of tests with acute and convalescent sera from case No. 2 (L) against Germiston and Bunyamwera viruses or antigens therefrom*

Serum	Virus					
	Bunyamwera			Germiston		
	HI	CF	NT	HI	CF	NT
Acute (Aug. 26, 1958)	0	0	0	0	0	0
Convalescent						
(Sept. 19, 1958)	0	0	0.3	60	8	2.4
(March 10, 1959)	0	0	0	80	0	3.2

* Results of HI, CF and NT tests in this table are expressed in the same manner as those in Table 5.

valescent serum showed a protection test conversion to H 131, the virus strain isolated from her blood, and neutralized 3.7 logs of AR 1057 virus.

The results of HI, CF and NT tests with the acute and two convalescent sera from the donor of H 131 virus strain against Bunyamwera and Germiston (AR 1050) antigens or viruses are shown in Table 6. A significant monotypic immune response to Germiston virus was detected by each of the serological techniques.

DISCUSSION

The results of serological studies indicate that AR 1050 and AR 1057 viruses are closely related or identical and are thus both strains of Germiston virus. Although some overlap was noted with Bunyamwera virus, significant immunological differences have been shown to exist between that agent and Germiston virus. In recent studies Casals and Whitman[4] have described a new antigenic group of arthropod-borne viruses which they refer to as the Bunyamwera group. The results of immunological studies with Germiston virus would suggest that this agent belongs to the Bunyamwera group. This antigenic relationship between members of the various arthropod-borne virus groups[1] has been the basis for studies to determine possible methods of broad-spectrum prophylaxis in man.[7, 15, 12] In the present report the immunological overlap between Bunyamwera and Germiston viruses has been demonstrated, but infection in man with the former agent failed to prevent overt disease with the latter. There is thus suggestive evidence that immunization of man with a less virulent member of a g.oup will not induce effective immunity against more virulent antigenically heterologous members of the same group.

The degree of detectable antigenic relationship between Germiston and Bunyamwera viruses varied with the type of serological test and, to some extent, according to the manner and host in which the antibody had been evoked. In intracerebral adult mouse cross-neutralization tests, immune sera from laboratory animals (monkey and guinea pig) did not clearly show the difference in the antigenic make-up between the two viruses. In HI tests the immune sera from the same animals showed that there are measurable antigenic differences between the two viruses. However, in cross-CF tests using mouse hyperimmune sera, the differences between Bunyamwera and Germiston viruses are both quantitative and qualitative. Thus, unlike the relationships in groups A and B in which the two in vitro techniques are more sensitive than the intracerebral adult mouse neutralization test in detecting serological overlap, the group relationship between Germiston and Bunyamwera viruses has been more apparent through the use of the latter test.

The results of serological tests with sera obtained from the second case (L.), in which the diagnosis of Germiston virus infection was established by virus isolation, gave only a monotypic antibody response. Similarly, in the other case (K.), the evidence of CF, HI and NT tests for Bunyamwera virus infection was related only to that agent and not to Germiston virus. These differences in host response (laboratory animals versus man) have previously been noted and discussed with reference to Pongola and Bwamba fever viruses.[10] This report likewise tends to support the view that differences in antibody pattern evoked by single response to either Germiston virus or Bunyamwera virus are dependent on host species in addition to such factors as dose, route of inoculation and time of postinoculation sampling.

Although the AR 1050 strain of Germiston virus was isolated from a mixed pool of 5 *Culex theileri* and 12 *C. rubinotus*, it is interesting to note that the AR 1057 strain came from a pure pool of *C. rubinotus*. The significance of *C. rubinotus* as a host of arthropod-borne viruses is further evidenced by the isolation of two addi-

tional virus types from pools of this mosquito species collected during April 1958. One virus type was derived from a collection made elsewhere near Johannesburg[18] and the other from a collection made at Germiston Lake.[12]

It is interesting to speculate on the mode of appearance of the three viral strains isolated from mosquitoes collected at Germiston Lake. As can be seen from Figure 1, this is a body of water approximately 1 mile in length and ½ mile in width, situated in the middle of an urbanized industrial community. Along one margin of the lake is a well-kept recreational park and on a portion of another there is a golf course. Dense reeds extend 20 to 50 yards into the water along another margin of the lake. In a limited section of these reeds adjacent to a portion of the golf course, there was a heronry with hundreds of cattle egrets, *Bubulcus ibis* (L.). In addition, there were a few other avian species such as black-headed herons, sacred ibises, weavers, moorhens, reed warblers and doves. Adjacent to this section of reeds, collections of mosquitoes were made as they came to bite man. Considering the "animal barrier" afforded by the lake, cleared park and golf course, one is led to suspect the role of birds in introducing the viruses which were found there.

The isolation of three virus strains from mosquitoes collected at Germiston Lake did not resolve the problem which caused the work to be undertaken, namely, determination of the etiology of febrile illness in human beings at Germiston. Nevertheless, it is apparent that Germiston virus is pathogenic for man and that laboratory personnel working with this agent are particularly vulnerable to infection.

SUMMARY

1. Two strains of filterable virus were isolated from culicine mosquitoes collected at Germiston, Union of South Africa. One was derived from a mixed pool of 5 *Culex theileri* and 12 *C. rubinotus* and the other from a pool of 11 *C. rubinotus*. Results of tests reported herein indicate that the two strains are antigenically indistinguishable and the name Germiston virus has been applied in reference to the locality of origin.

2. Germiston virus is related to but not identical with Bunyamwera virus. With immune sera obtained after inoculation of laboratory animals, antigenic differences between the two viruses were easily demonstrated by HI and CF tests, less easily by neutralization test.

3. Mice immunized with Bunyamwera were not significantly protected when challenged with Germiston virus.

4. Two clinical cases of Germiston virus infection occurred in laboratory workers. Even though a significant level of Bunyamwera virus neutralizing antibody was present in sera of one of the individuals within only a few hours after onset and also in a specimen taken approximately 7 months previously, infection with Germiston virus was not prevented. The implications of this observation and results of other tests on sera from this case obtained at various time intervals are discussed.

5. Observations are presented which lead to the suspicion that birds may have had a role in infecting mosquitoes with Germiston virus.

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KAIRI VIRUS, A NEW VIRUS FROM TRINIDADIAN FOREST MOSQUITOES*

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During the course of arthropod-borne (arbor) virus investigations in Trinidadian forests in 1955, 31 viruses were isolated in baby mice from mosquitoes taken while attacking human bait. Of these, 27 came from the Melajo forest, 7½ miles from Sangre Grande in the northeastern part of the island. Ten of the Melajo agents have been shown to be strains of the same virus, a virus which is apparently new to science and for which the name Kairi (meaning "island" in Carib dialect) is proposed. An eleventh strain of the same virus was recovered in 1958 from the Archer Estate near Rio Claro in South Trinidad.

ISOLATION

Table 1 presents the pertinent information concerning the various mosquito suspensions from which the strains of Kairi virus were isolated. It is of interest that four of the strains came from pools of *Aedes scapularis*, four from *Wyeomyia* pools (including *W. aporonoma* and *W. ypsipola*), two from pools of *Psorophora ferox* and one from *Culex spissipes*. All of the 1955 strains were isolated within a span of 7 weeks during August and September; the 1958 strain was also isolated in August.

The mosquitoes were ground with alundum in a mortar and suspended in 2 ml of diluent† with the exception of the two *P. ferox* pools, which were suspended in 3 ml. The suspension was then centrifuged for ½ hour in an angle centrifuge at about 8,000 rpm in a 4°C cold room. The supernatant was inoculated intracerebrally into a group of 2-day-old mice. An aliquot was stored in a sealed glass ampoule in the CO_2 ice chest.

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† The diluent employed was phosphate buffered 0.75% bovalbumin in normal saline which contained 400 units of penicillin and 250 μg of streptomycin per ml. This diluent was sterilized by Seitz filtration.

The course of the adaptation of the various strains to mice followed a consistent pattern. In fact, this similarity was the first clue to possible identity of the strains. Most of the mice inoculated with the original mosquito suspensions showed signs of illness between the 6th and 10th postinoculation days. Nine of the strains were isolated from passages of mice which sickened within that interval. Of the remaining two strains, one was isolated from a mouse sick on the third postinoculation day and the other from a mouse sick on the 12th postinoculation day. During the succeeding passages in infant mice, the infection increased in virulence and decreased in length of incubation period, with the result that by the fourth passage all strains were killing mice in from 3 to 5 days following intracerebral inoculation of infected brain suspensions.

About 3 years after isolation, an attempt was made to reisolate virus from the stored mosquito suspensions from the following specimens: 8900, 9027, 9093, 9148, 9424 and 9638. Virus was reisolated from all but 9148. A careful review of the isolation of that strain disclosed no reason why its authenticity should be doubted.

It was also characteristic that adult mice showed no signs of infection following intracerebral inoculation of early passage infant-mouse brain. However, after a few baby-mouse passages, the strains became sufficiently well adapted to mice to produce signs of illness when inoculated intracerebrally into adult mice. Further intracerebral brain passages increased the virulence of the strains to adult mice to the point where the mice regularly died in from 4 to 6 days after inoculation.

There was nothing noteworthy about the illness produced by Kairi virus in either adult or infant mice. Paralysis was not a prominent sign. The mice generally went through a period of irritability, which was followed by loss of appetite, prostration and death. The adult mice showed ruffling of the coat. After a few passages the strains were almost as virulent by the intraperitoneal as by the intracerebral route in infant

TABLE 1

Composition of various mosquito pools from which strains of Kairi virus were isolated

TRVL No.	Species of mosquito	Place	Date of collection	No. mosquitoes in pool
8900	*Aedes scapularis*	Melajo forest	Aug. 9, 1955	285
9027	*Aedes scapularis*	Melajo forest	Aug. 16, 1955	358
9093	*Wyeomyia* pool*	Melajo forest	Aug. 18, 1955	206
9148	*Aedes scapularis*	Melajo forest	Aug. 22 and 23, 1955	312
9412	*Psorophora ferox*	Melajo forest	Sept. 7, 1955	359
9424	*Aedes scapularis*	Melajo forest	Sept. 8, 1955	140
9467	*Wyeomyia* pool*	Melajo forest	Sept. 9, 1955	223
9528	*Culex (Melanoconion) spissipes*	Melajo forest	Sept. 12 and 13, 1955	12
9638	*Psorophora ferox*	Melajo forest	Sept. 19, 1955	748
9688	*Wyeomyia aporonoma*	Melajo forest	Sept. 21, 1955	103
22230	*Wyeomyia ypsipola*	Archer Estate	Aug. 6, 1958	92

* Species in this pool undetermined.

mice. Intraperitoneal inoculation of adult mice did not produce visible signs of infection. The titer of virus in adult mice inoculated intracerebrally was generally not as high as the titer in baby mice by the same route. The titer of baby-mouse brain suspension in baby mice was approximately 10^{-6} LD_{50}/0.02 ml.

Attempts were made to passage three strains of Kairi virus (8900, 9027, 9424) in chick embryos by the yolk, allantoic and amniotic sac routes. No virus was detected in any of the subsequent passages, although some of the eggs in the original groups contained virus when tested in mice.

An attempt to propagate the 8900 strain in hamster kidney culture was unsuccessful.

IDENTIFICATION

From the behavior of these strains in mice it was quite clear that they were not the members of either Group A (Venezuelan and eastern equine encephalitis and Mayaro) or Group B (yellow fever, dengue, St. Louis and Ilhéus) then known to be, or suspected of being, in Trinidad. Consequently, one strain, TRVL 8900, was sent to The Rockefeller Foundation Virus Laboratories in New York for further study.

The studies done at that time in New York, which were not exhaustive, failed to turn up any lead as to the identity of the virus. It was not possible to prepare a hemagglutinating antigen. A suitable complement-fixing antigen was prepared which reacted with a homologous mouse hyperimmune serum. This mouse hyperimmune serum failed to inhibit hemagglutination by Group A and Group B viruses. It also failed to inhibit hemagglutination by a Bunyamwera antigen.

Meanwhile, in Trinidad, mouse immune sera to representative strains of virus isolated in 1955 had been prepared. These sera were tested in neutralization tests against the several viruses as well as against other strains isolated at that time in an attempt to discover antigenic relationships. The results showed the following strains to be related: 8900, 9027, 9093, 9148, 9412, 9424 and 9638.

The 8900 strain has been compared with three other strains of Kairi virus by determination of neutralization indices. The sera employed in these tests were prepared from adult mice inoculated intracerebrally with early mouse-brain passage material. Serial 10-fold dilutions of the infected brain suspension were incubated for an hour at 37°C with an equal volume of known normal or immune mouse serum and inoculated

TABLE 2

Cross-neutralization tests between the 8900 strain and three other strains of Kairi virus

Virus	Serum			
	8900	9093	9424	9638
8900	4.3*	>4.2	2.4, 2.7*†	3.4
9093	4.7	>3.2		
9424	>3.5		>4.5	
9638	3.8, 3.3†			>4.6

* No. of logs LD_{50} of virus neutralized.

† Result of a repeat test with the same serum.

intracerebrally into either adult or infant mice. (Previous tests had revealed very little difference in the immune sera's neutralizing capacities when tested in either adult or infant mice by the intracerebral route.) The results in Table 2 show the close relationship existing between the strains. The 8900 virus mouse immune serum also neutralized >4.6, >3.7, and >3.2 logs LD_{50}, respectively, of 9027, 9148 and 9412 strains of Kairi virus.

Three strains—9093, 9424 and 9638—were studied in cross-complement-fixation tests by the technique described by Casals and Palacios.[1] It was found that these strains were as closely related by CF tests as they were by neutralization test. Four other strains have been identified by CF test alone: 9467, 9528, 9688 and 22230. The validity of the CF test for the identification was based on the observation that an 8900 antigen failed to react in CF test with mouse hyperimmune sera prepared from the following antigens: Russian spring-summer, West Nile (Egypt), Japanese B, Ntaya, Zika, Uganda S (Makonde), California Bat (M 64), mouse encephalomyelitis (GD VII), Sindbis, Chikungunya, Semliki Forest, eastern and western equine encephalitis, California, Simbu (TAR 53), Spondweni (TAR 94), Be H 151 (Belém), Be H 5056 (Belém), Colombia 35211, sandfly fever (Naples), Be An 73 (Belém), Ar 1095 (Egypt), Ar 1152 (Egypt), Anopheles A, Ar 492 (Egypt), Turlock (781-19), Wyeomyia, Bwamba, Rift Valley fever (Lunyo), Oriboca (Be An 17), Marituba (Be H 176), Apeu (Be An 848) and Mengo. A Bunyamwera hyperimmune serum gave a positive reaction in 1:2 dilution. The relationship of Kairi virus (8900) to the Bunyamwera Group was discovered at The Rockefeller Foundation laboratories in New York and has been described in detail in a paper by Casals and Whitman.[2]

All of the strains passed readily through a bacteria-tight Seitz-EK pad. The 8900 strain has been found to be inactivated by sodium desoxycholate.[3]

SERUM SURVEY

A group of 63 human sera obtained from Trinidadians with a history of prolonged forest exposure was tested against the 8900 strain in a standard adult mouse intracerebral neutralization test employing 631 LD_{50}. Only one serum showed any evidence of virus neutralization.

When retested in an intraperitoneal baby-mouse test, this serum gave full protection. Casals and Whitman have pointed out that Cache Valley[4] and Wyeomyia immune sera neutralize significant amounts of Kairi virus when tested in the baby-mouse intraperitoneal test. They also state that there is even evidence of protection in the intracerebral test. Unpublished data from Trinidad have indicated the presence on the island of at least one other member of the Bunyamwera Group. Under the circumstances the present result cannot be considered as indicating human infection by Kairi virus. The antibodies may have been produced as the result of infection by a closely related virus.

A group of Trinidadian howler and *Cebus* monkey sera was tested in an 8900 virus adult-mouse intracerebral neutralization test employing 741 LD_{50}. Two of a group of 34 howler monkey sera showed a partially protective reaction. Again, the significance of the reaction is in doubt, in that it is not possible to determine whether the result indicates infection by Kairi virus or by some closely related virus. One of a group of 18 *Cebus* monkey sera completely neutralized the virus.

A group of 19 Trinidad donkey sera were tested in the baby-mouse intraperitoneal test. Eleven of the sera contained neutralizing antibodies. Again, a categorical interpretation is rendered difficult by the possibility of cross-reactions with related viruses, since the test dose employed was only 100 LD_{50}.

SUMMARY

The isolation of a new virus from several species of Trinidadian mosquitoes has been described. The virus has been named "Kairi virus." The presence of virus-neutralizing antibodies in the sera of human beings and several species of animals of Trinidad was detected.

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A NEW ANTIGENIC GROUP OF ARTHROPOD-BORNE VIRUSES

The Bunyamwera Group

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Immunological cross-reactions among arthropod-borne (arbor) viruses are the basis for a classification of these agents; three distinct antigenic groups have already been reported.[1] It is the purpose of this paper to describe a new set of immunological cross-reactions which have resulted in the assembling of four previously unclassified viruses in a hitherto unrecognized group, for which the designation Bunyamwera Group is proposed.

MATERIALS AND METHODS

Viruses. The viruses shown to be related by the present study are the following:

Bunyamwera, isolated and characterized by Smithburn *et al.*[2] This virus was derived from a mixed pool of *Aedes* mosquitoes captured in Uganda, East Africa.

Wyeomyia, isolated by Roca-Garcia[3] from mosquitoes, *Wyeomyia melanocephala*, in Colombia.

Cache Valley virus, a new virus recently isolated by Drs. Preston Holden and A. D. Hess (Encephalitis Investigations Unit, Greeley, Colorado) from mosquitoes, *Culiseta inornata*, trapped in Utah and named by them. The strain which Dr. Holden made available to us, 6V-633, is described elsewhere.[4]

Kairi virus, a new virus isolated by Dr. C. R. Anderson *et al.* and to be described by them.[5] Strain Tr 8900 of this virus was isolated from mosquitoes, *Aedes scapularis*, caught in Trinidad, W. I., and as with Cache Valley virus, the immunological studies leading to its characterization were done in part in this laboratory.

Two additional viruses were included in the investigation, both isolated by Dr. O. R. Causey from pools of mosquitoes, *Aedes scapularis*, caught in the forest near Belém (Pará), Brazil. During the course of the studies the first of these viruses, Be Ar 7272, was found to be serologically indistinguishable from strain 6V-633 and was therefore considered as another isolation of Cache Valley virus. The second virus, Be Ar 8226, appears indistinguishable from Tr 8900 and is treated here as a second strain of Kairi virus.

Serological Tests. The relationships between the four viruses were studied by complement-fixation (CF), neutralization and hemagglutination-inhibition (HI) tests.

In the CF tests, the antigens were prepared from brain tissue of infected newborn mice, extracted with acetone either directly or after homogenization in a solution of sucrose. The procedure of simultaneous testing of serial dilutions of both antigen and serum, box titration, was used extensively.

In the neutralization tests, mixtures of equal parts of undiluted serum and virus in increasing 10-fold dilutions were incubated at 37°C for 1 hour, then inoculated into mice. The route of inoculation was either intracerebral or intraperitoneal; the former was used in attempts to separate closely related viruses, the latter for demonstration of overlap between distantly related agents. Depending on the pathogenicity of the virus and on the route of inoculation, the mice used were either newborn or weanlings.

The methods for the HI tests have been described in detail.[6] Of the six strains mentioned, only Bunyamwera and Be Ar 7272 have so far yielded agglutinating antigens; these were prepared by the method of precipitation with acetone of a suspension of infected brain tissue made in a solution of sucrose.

Immune Sera. Most of the sera used were derived from mice that had received from four to six intraperitoneal injections; previous experience with the other arbor virus groups[1] had shown that multiple injections generally result in sera which have a wider range of cross-reactions and, hence, are suitable for establishing groups. Between collection and testing, the sera were stored at −20°C.

RESULTS

To establish a new antigenic group, it is necessary to show that cross-reactions exist between

TABLE 1

Complement-fixation tests with mouse hyperimmune sera

Antigen	Serum			
	Bunyamwera	Cache Valley 6V-633	Kairi Tr 8900	Wyeomyia
Bunyamwera	*128/512	64/512	16/512	8/512
Cache Valley, 6V-633	64/64	128/128	16/128	8/128
Kairi, Tr 8900	4/4	0/0	256+/64	2/2
Wyeomyia	0/0, 2/4	0/0	0/0	64/128

* 128/512 = Titer of serum 1:128, titer of antigen 1:512.

the members of the new group and that no cross-reactions can be found between any of the members of the new group and viruses of previously described groups.

Cross-Reactions in CF Tests. In a series of tests in which the antigens and antisera of the four virus entities were used interactively, a group relationship became evident. A typical example of such tests is given in Table 1. In this table, the results of box-titration tests are expressed as a ratio, the maximal titer of the serum being the numerator and the maximal titer of the antigen in the same box-titration, the denominator. As shown in the table, the antigens of Bunyamwera and Cache Valley virus will react not only with their own sera, but also with the sera from the other members of the group. Furthermore, this antigen reactivity is of more or less equal sensitivity regardless of the specificity of the serum. In contrast, both Kairi Tr 8900 and Wyeomyia antigens seem to be quite individual, either not reacting at all with the heterologous sera or, at best, reacting minimally when an excess of both antigen and serum is combined. This failure of Kairi and Wyeomyia antigens to cross-react is basically responsible for the delay in recognition of the immunological relationships between the members of this group.

In Table 1, it can be seen that while an excess of both Kairi (strain Tr 8900) and Wyeomyia sera is required before these will react with either Bunyamwera or Cache Valley (strain 6V-633) antigens, the two latter and their sera cross-react almost to titer. There are, however, slight differences in that both Bunyamwera and Cache Valley sera are active in a 2-fold higher dilution with the homologous antigen than with the heterologous antigen. These differences, although minimal, have been observed with reasonable consistency. When it was found that Be Ar 7272 belonged with the new group of agents, in particular with the Bunyamwera-Cache Valley members, similar cross-reactions were studied. An example is given in Table 2. Again, slight differences can be observed. Both Cache Valley (strain 6V-633) serum and Be Ar 7272 serum are 2- to 4-fold more reactive with their homologous antigens than with the Bunyamwera antigen, while Bunyamwera serum is 2-fold less reactive to the Be Ar 7272 antigen than to its own. Even this amount of difference, however, is not observable between 6V-633 and Be Ar 7272, a fact suggesting that perhaps these two are truly identical.

As stated in the introduction, Be Ar 8226 virus was received for study at the same time as Be Ar 7272. In CF tests, it was found to be indistinguishable from Kairi strain Tr 8900. An example of repeated cross-tests is also included in Table 2. It is of interest to note that while Kairi strain Tr 8900 antiserum, which had a

TABLE 2

Complement-fixation tests with mouse hyperimmune sera

Antigen	Serum				
	Bunyamwera	Cache Valley		Kairi	
		6V-633	Be Ar 7272	Tr 8900	Be Ar 8226
Bunyamwera	128/512	64/512	64/512	16/512	0/0
Cache Valley, 6V-633	128/128	128/128	256/256	8/64	0/0
Cache Valley, Be Ar 7272	64/512	128/512	256/512	8/256	0/0
Kairi, Tr 8900	8/32	0/0	0/0	256/64	64/64
Kairi, Be Ar 8226	16/128	4/8	4/32	256/128	64/128

TABLE 3

Neutralization tests with mouse hyperimmune sera, log neutralization indices

Serum	Virus and Route of Inoculation				
	Intraperitoneal		Intracerebral		
	Bunyamwera*	Kairi, Tr 8900	Bunyamwera	Cache Valley, 6V-633	Kairi, Be Ar 8226
Bunyamwera	≷5.0	≷3.9	4.4	3.0	1.9
Cache Valley, 6V-633	3.9	≷3.9	2.3	≷4.2	1.7
Kairi, Tr 8900		≷3.6			≷3.9
Kairi, Be Ar 8226					≷3.9
Wyeomyia	3.3	3.0			1.8
Controls†	(1) 0.5 (2) 0.0 (3) 0.3 (4) 0.7	(3) 0.1 (5) −0.4	(3) 0.4		(4) 0.3 (6) 0.1 (7) 0.0 (8) 0.5

* Mice of 3 to 4 weeks of age used in this test. In all other tests, the mice were 3 to 4 days of age at the time of inoculation.

† In addition to normal sera, hyperimmune sera against the following viruses were included: (1) Simbu, (2) Tr 9760, (3) St. Louis, (4) California encephalitis, (5) Bwamba, (6) Be An 7882, (7) Be Ar 8033, and (8) Guaroa.

4-fold higher titer than Be Ar 8226 serum, reacted with Bunyamwera, 6V-633 and Be Ar 7272 antigens, Be Ar 8226 serum did not. Similarly, Be Ar 8226 antigen had a 2-fold higher titer than Kairi Tr 8900 antigen and was more reactive with the heterologous sera.

Cross-Neutralization Tests. In order to confirm the demonstrable CF relationships, neutralization tests were undertaken. To intensify group overlap, two tests were done using the intraperitoneal route of inoculation; in the remaining tests the intracerebral route of inoculation was used in an attempt to develop specific differences. The pertinent data are shown in Table 3. In the intraperitoneal tests, it is clear that Cache Valley serum and Wyeomyia serum neutralize both Bunyamwera and Kairi strain Tr 8900 viruses to a significant degree. Even in the intracerebral tests, there is evidence of group relationship. However, the results indicate a lack of identity between Bunyamwera and Cache Valley virus in that the respective sera neutralize their own viruses to a greater extent than they neutralize the other. The results with Kairi strain Be Ar 8226 tend to confirm the identity of this agent with Kairi Tr 8900 and, at the same time, show that the sera from the other group members are capable of at least detectable virus neutralization even by this route.

Cross-Reactions in HI Tests. Before Be Ar 7272 was received, the only member of the group from which a hemagglutinin could be prepared was Bunyamwera. Using this antigen, repeated attempts had been made to demonstrate inhibition with several heterologous sera but with generally negative results. When four different specimens of immune sera for each of the several viruses were used, each on several occasions, inhibition was infrequently observed and then only in low dilution. In these tests, Bunyamwera sera were capable of inhibiting 4 or 8 units of the antigen over a range of dilutions from 1:160 to 1:2560. In 16 such tests with Cache Valley sera, no inhibition was observed on 12 occasions; in 3, inhibition occurred at dilution 1:10 (the lowest used), and in one, at dilution 1:20. In four tests with Tr 8900 sera, three were negative, and one inhibited at dilution 1:10. Finally, 13 of 14 tests with Wyeomyia sera were negative and one inhibited in dilution 1:10.

With the preparation of a hemagglutinin from Be Ar 7272, it became possible to study the cross-reactivity of the group to a second antigen. It was immediately found that while Cache Valley sera (strain 6V-633) would readily inhibit this antigen, the other sera were either nonreactive or reacted only to the same limited extent observed in cross-reactions with Bunyamwera hemagglutinin. The results of a comparative test are given in Table 4. In this experiment, three 2-fold dilutions of each test hemag-

TABLE 4

Hemagglutination-inhibition tests

Sera	Antigens and Units					
	Bunyamwera			Cache Valley, Be Ar 7272		
	8	4	2	8	4	2
Bunyamwera						
a	80*	160	640	0	0	0
b	320	1280	5120	0	10	20
Cache Valley, 6V-633						
c	0	0	0	80	320	640
d	0	0	0	160	320	640
Cache Valley, Be Ar 7272						
e	0	10	10	640	1280	2560
f	0	0	0	160	320	320
g	0	0	0	160	320	320
h	10	10	20	40	80	160
Kairi, Tr 8900						
i	0	0	0	0	0	10
j	10	10	40	0	0	20
Kairi, Be Ar 8226						
k	0	0	0	0	0	0
l	0	0	0	0	0	0
Wyeomyia						
m	0	0	0	0	0	0
n	0	0	0	0	0	10

* 80 = reciprocal of the highest serum dilution inhibiting hemagglutination. 0 = no inhibition at dilution 1:10.

glutinin were used, containing respectively 8, 4 and 2 hemagglutinating units. As can be seen, 6V-633 and Be Ar 7272 sera inhibited the Be Ar 7272 antigen in equally high dilution. This result gives still further evidence of the close similarity, if not identity, of these two agents. The Bunyamwera serum inhibited its own antigen. Otherwise, cross-reactions were minimal.

Absence of Cross-Reactions with Viruses of Groups A, B and C. Negative evidence necessary to define the present Bunyamwera Group was gathered partly from published reports, but to a greater extent during the present investigation. In the course of the studies done to characterize Bunyamwera[2] and Wyeomyia[3] viruses, as well as in subsequent work by Smithburn[7, 8] and Kerr,[9] no cross-reactions were found by neutralization test—mainly intracerebral inoculation—or CF test between these two viruses and several others which were subsequently placed in groups A or B. The extensive work done in this laboratory, mainly by HI and CF, that resulted in the recognition of antigenic groups of arbor viruses likewise failed to uncover any evidence of serological overlap between Bunyamwera or Wyeomyia and any of the viruses in groups A, B and C.

During the characterization of Cache Valley and Kairi viruses in this laboratory, they were compared, by CF and HI, with a variety of viruses of groups A, B and C. The discovery of the present new group also prompted a reinvestigation of the relationship of Bunyamwera and Wyeomyia viruses to viruses of the other groups. The results of these studies can be summarized as follows. No cross-reactions have been found by CF between immune sera to any of the four Bunyamwera Group viruses and antigens from western equine encephalitis, AMM 2021,* AMM 2354* and Sindbis viruses (group A) and St. Louis encephalitis virus (group B); nor has western equine encephalitis immune serum reacted with any Bunyamwera Group antigens. Immune serum to Apeu (group C) failed to react with Cache Valley virus antigen. In HI tests, immune sera to any of the present Bunyamwera Group viruses did not inhibit group A antigens, Chikungunya and eastern equine encephalitis, or group B antigens, dengue type 2, Japanese encephalitis, Ilheus, St. Louis encephalitis and yellow fever. Also, Bunyamwera immune sera did not inhibit group C antigens, Oriboca and Marituba, nor did Kairi serum inhibit Oriboca antigen. Finally, no reaction was found between Bunyamwera antigen and immune sera to Mayaro, Semliki, Sindbis, western equine encephalitis (group A), Japanese encephalitis, Ilheus, St. Louis encephalitis, West Nile (group B), or Oriboca (group C) viruses.

DISCUSSION

That the four virus entities included in the new group are immunologically related is beyond question. It must be emphasized, however, that the demonstration of this fact has required the use of both strong antigen systems and hyperimmune sera. Although the CF relationship between Bunyamwera and Cache Valley virus is easily detectable with average reagents, early studies

* AMM 2021 and AMM 2354 are group A viruses isolated by Dr. E. L. Buescher.

failed to reveal the connection between Kairi and Wyeomyia, or between these two viruses and Bunyamwera. In the previous groups described (A, B and C), the group reactivity as demonstrated in HI tests has been sufficiently marked to suggest the group placement of unknown viruses quickly, and the problem has more often been that of determining the degree of difference between a new agent under study and the known members of the group. In the Bunyamwera Group, there is no such readily demonstrable common denominator, and of the tests available, HI has been so far the least helpful.

SUMMARY

Four arthropod-borne viruses—Bunyamwera and Wyeomyia, both in the literature for many years, and Cache Valley and Kairi viruses, newly discovered—have been found to be related serologically among themselves and not to viruses in arbor virus groups A, B and C. This finding fulfills the criteria for the establishment of a new antigenic group in the arbor viruses. In the past, such groups have been designated alphabetically; however, as already indicated,[10] this type of nomenclature may, with increasing number of groups, cease to be practical, and furthermore, may result in conflicting designations by independent investigators. For these reasons, it is considered advisable, at least pending final solution of the problem of nomenclature of arthropod-borne virus groups, to designate this new group by the name of one of its members. Accordingly, the designation Bunyamwera Group has been selected.

ADDENDUM

Since the data on which this paper is based were obtained, we have received for study other virus isolates from Africa, India and South America which belong to this new group but appear to have sufficient individuality to justify consideration as specific entities. One of these, isolated and described by Kokernot *et al.*[11] in South Africa, has been designated Germiston virus. In addition, we have evidence that there may be differences among the several strains of Wyeomyia virus under study which eventually may justify further subgrouping. It is anticipated that the Bunyamwera Group will expand rapidly.

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MANZANILLA VIRUS: A NEW VIRUS ISOLATED FROM THE BLOOD OF A HOWLER MONKEY IN TRINIDAD, W. I.*

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During the yellow fever epidemic in Trinidad in 1954 a considerable number of howler monkeys (*Alouatta seniculus insularis* Elliot) were shot in the course of investigative work.[1] At the time of collection of sera from these animals, deaths were occurring frequently in howlers, and accordingly the sera were routinely inoculated into baby white mice in the hope of isolating yellow fever virus. Sixty-eight sera were tested but yellow fever virus was recovered from none. However, from the serum of one monkey a virus was isolated which has not been identified with any of the known viruses. This agent has been named "Manzanilla virus" since the monkey was from the Manzanilla region of Trinidad.

ISOLATION

On June 16, 1954, a sample of heart's blood (TRVL Sp. No. 3587) was obtained from a howler monkey shot at Brigand Hill, near Manzanilla. Brigand Hill is a hilly forest bordering the northern limits of the Nariva swamp near the east coast of Trinidad. Serum specimens were also obtained from six other monkeys of the same band. These specimens were refrigerated immediately after being taken, and a few hours later, at the laboratory, were inoculated intracerebrally into 2-day-old mice. An aliquot was stored in a sealed glass ampoule in the CO_2 ice box. The remainder of the serum was stored at −20°C in a sterile lusteroid tube. Ten days after inoculation a mouse was found sick and a suspension of its brain was passed intracerebrally to another group of infant mice. The mice in this group were all dead by the third postinoculation day and thereafter the agent was easily maintained in intracerebral passage in either adult or baby mice. The virus was successfully filtered through a bacteria-tight Seitz-EK pad. As only one mouse in the original group had sickened, it was anticipated that there would be difficulty in reisolating the virus from the sample stored in the CO_2 ice box. Accordingly, six groups of 2-day-old mice were inoculated with the stored serum, the sample then being exhausted. Almost all of the mice inoculated succumbed to the infection and the virus was recovered from several of them.

IDENTIFICATION

Specific immune sera to the following viruses have been tested for their ability to neutralize Manzanilla virus: eastern, western and Venezuelan equine encephalitis, St. Louis, yellow fever, Semliki Forest, Bunyamwera, Ilhéus and Mengo. None of the sera showed significant neutralization of the virus.

In a complement-fixation (CF) test, a mouse-brain antigen prepared from TRVL 3587 gave a positive reaction with a homologous mouse hyperimmune serum. The titer for both antigen and serum was 1:128. This antigen did not react in CF tests with mouse hyperimmune sera prepared from each of the following viruses: West Nile (Egypt 101 strain), dengue (Hawaiian and Trinidad 1751 strains), Russian spring-summer encephalitis, St. Louis, yellow fever, Zika, Japanese B, Ilhéus, eastern, western and Venezuelan equine encephalitis, Semliki Forest, Bunyamwera, Wyeomyia, Kairi (Trinidad 8900 strain),[2] Bwamba, California, sand-fly fever (Naples strain), Rift Valley fever (Lunyo strain), Oriboca (BeAn 17) and mouse encephalomyelitis. The antigen likewise failed to react in CF tests with mouse hyperimmune sera prepared from each of a group of unidentified viruses isolated by Rockefeller Foundation workers in Brazil, South Africa and Trinidad: Be An 15, Be An 73, Be An 277, Be H 151, SA-TAR 53 and TRVL 9760.

Attempts to prepare a viral hemagglutinin by acetone-ether extraction were unsuccessful. Dr. Akira Oya of the National Institute of Hygiene, Tokyo, a guest worker at the Trinidad Regional Virus Laboratory, prepared a hemagglutinating

* The studies and observations upon which this paper is based were conducted by the Trinidad Regional Virus Laboratory with the support and under the auspices of the Government of Trinidad and Tobago, the Colonial Development and Welfare Scheme and The Rockefeller Foundation.

antigen by the sucrose-acetone method described by Clarke and Casals.[3] A crude borate-saline buffered hemagglutinin of low titer was also prepared. The sucrose-acetone antigen was inhibited by the homologous hyperimmune mouse serum but was not inhibited by hyperimmune mouse sera prepared from a Group A virus (Mayaro) and two Group B viruses (Ilhéus and St. Louis). Mouse hyperimmune serum prepared from Manzanilla virus did not inhibit hemagglutination by Mayaro, Ilhéus and St. Louis antigens.

Manzanilla virus was inactivated by sodium desoxycholate under the conditions prescribed by Theiler.[4]

REACTION OF VARIOUS ANIMALS AND CHICK EMBRYOS TO MANZANILLA VIRUS

White Mice. Two-day-old mice have been found to be susceptible to Manzanilla virus inoculated either intraperitoneally or intracerebrally. The incubation period following intracerebral inoculation was from 2 to 6 days, depending on the size of the dose; that following intraperitoneal inoculation was from 3 to 8 days. When the titers of suspensions were compared by the two routes it was found that the titer following intracerebral inoculation (10^{-5} to 10^{-7}/0.03 ml) was slightly higher than that following intraperitoneal inoculation (10^{-4} to 10^{-6}/0.03 ml). Virus was found on the second postinoculation day in the blood of 2-day-old mice inoculated intraperitoneally but was not detected in the blood following intracerebral inoculation.

Adult mice were susceptible to Manzanilla virus inoculated intracerebrally but failed to show any visible signs of infection after intraperitoneal inoculation of about 10^6 intracerebral LD_{50}. The incubation period in adult mice was from 3 to 7 or 8 days. The only unusual sign of disease in the adult mice was the tendency of the sick mice to develop convulsive seizures when the cage was picked up or they were stroked on the back. During a seizure a mouse would carom about the cage for a few seconds, its body in a flexed position, and afterwards lie exhausted and prostrate for several minutes. The titer of virus in adult mouse brain was 10^{-5} to 10^{-6} LD_{50}/0.03 ml.

Chick Embryos. Chick embryos have been found to be highly susceptible to Manzanilla virus when inoculated by the yolk sac, amniotic sac or allantoic sac routes. The virus was maintained in continuous passage for a total of 34 passages by the inoculation of embryo suspensions into the yolk sac. The incubation period was from 3 to 7 days, depending on the dose. The titer of virus in the embryo was about 10^{-7} LD_{50}/0.1 ml.

Day-old Chicks. Day-old chicks inoculated intracerebrally with about 1,000 mouse LD_{50}'s showed no visible signs of infection and did not circulate virus. Neutralizing antibody was not detected in serum samples obtained 26 days later.

Rabbits. No visible signs of illness occurred in adult rabbits receiving several thousand mouse LD_{50}'s by the intraperitoneal or intracerebral route of inoculation, and the animals did not circulate virus. Antibodies were not detected in the sera of any of the rabbits 39–47 days after inoculation.

Hamsters. Adult hamsters inoculated intracerebrally with Manzanilla virus regularly succumbed to the infection. The incubation period was from 5 to 8 days. The animals stopped eating, developed paralysis and generally died a day or so after the onset of illness. Brain suspensions from several sick hamsters were titered in mice and the highest titer observed was 10^{-5} LD_{50}/0.03 ml. When inoculated intraperitoneally, the hamsters showed no visible signs of infection but did develop demonstrable neutralizing antibodies. Circulating virus studies were not done.

Guinea Pigs. Adult guinea pigs inoculated with large doses of virus by either the intracerebral or intraperitoneal route failed to develop visible manifestations of infection. They did, however, produce virus-neutralizing antibodies.

ENTOMOLOGICAL STUDIES

A few attempts were made to transmit Manzanilla virus by allowing parenterally inoculated mosquitoes (after an incubation period of 2 weeks) to feed on day-old mice or pledgets of cotton soaked in sugar solution. These were all unsuccessful. The mosquitoes themselves, however, when subsequently tested, were shown to contain virus, as follows:

Aedes scapularis: pool of three mosquitoes positive after 2 weeks' extrinsic incubation.

Aedes serratus: one mosquito positive and pool of two mosquitoes positive after 2 weeks of incubation.

Culex fatigans: 13 mosquitoes negative at 2 weeks; one mosquito positive at 3 weeks.

The virus recovered from these mosquitoes was identified by complement fixation.

SERUM SURVEY

A group of 47 human sera was tested for the presence of Manzanilla-virus neutralizing antibodies. The donors, who came from representative forested areas in Trinidad, were heavily exposed to the bites of forest mosquitoes. None of these sera neutralized significant amounts of the virus.

A group of 67 howler monkey sera has also been tested for the presence of neutralizing antibodies. The monkeys furnishing the specimens were widely distributed in the forested regions of northeastern and eastern Trinidad. Eight of the group were considered to show virus-neutralizing antibodies. (The serum from the monkey from which the virus was isolated failed to neutralize the virus.) It was found that many of the sera contained antiviral substances which could be destroyed by treatment of the sera for 30 minutes at 56°C. The sera considered to contain neutralizing antibody were ones that neutralized virus after being treated at 56°C for 30 minutes.

Six sera which showed evidence of virus-neutralizing antibodies were tested for hemagglutination-inhibiting activity. None of the sera inhibited hemagglutination. The significance of this result, in combination with the evidence of virus neutralization, is difficult to evaluate until more has been learned about the infection in howler monkeys. From laboratory experiments it is known, however, that some viruses will stimulate the production of high-titer neutralizing antibodies while stimulating very low-titer and often transient hemagglutination-inhibiting antibodies.

Sera obtained from the local *Cebus* monkey were not tested because it was found that monkeys captured and tested in the laboratory were completely refractory to the virus inoculated either intraperitoneally or intracerebrally.

SUMMARY

The isolation and laboratory studies of an unidentified virus isolated from a howler monkey in Trinidad, W. I., have been described. The virus has been named "Manzanilla virus" after the region from which it was obtained. The absence of virus-neutralizing antibodies from the sera of a number of forest workers would seem to indicate that this virus probably is not a frequent cause of infection in man.

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ELECTROPHORETIC PATTERN OF HYPERPROTEINEMIC SERA IN A POPULATION GROUP OF RURAL PANAMA*

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Elevated serum protein levels of unknown etiology have been described in several specific population groups.[3, 6, 11, 14, 15] In the Central American area, hyperproteinemia is particularly common among adults in rural Panama.[11] The present study was designed to determine whether the increase of a particular serum protein fraction or fractions could account for these high values or whether all of the fractions were elevated proportionately. The results are pertinent to the basic problem of the mechanisms involved in the production of these hyperproteinemias.

MATERIALS

The study was conducted during May in the village of La Arena, Chitré, Panama, located at sea level approximately 5 kilometers from the Pacific Coast. The mean temperature was approximately 28° C. The subjects were an ethnic mixture of white, Negro and Indian, whose cultural characteristics and dietary habits have been carefully investigated.[13] The total consumption of protein was inadequate in at least 50% of the families studied, although calorie intakes closely approximated estimated requirements. About 33% of their dietary protein was of animal origin. Their average intake of riboflavin was 50% and that of calcium 34% of the recommended allowances for Central America and Panama.[8] No other dietary deficiencies of minerals or vitamins were found. Total serum proteins were determined in 98 individuals, 58 males and 40 females ranging in age from 13 to 66 years. To study the distribution of the serum protein fractions and its possible relation to the level of total proteins, 9 subsamples were analyzed by electrophoresis from three groups selected as follows: Group I: Total protein contents below the median; Group II: values between the median and the third quartile; Group III: protein levels above the third quartile. The latter represented unusually high serum total protein values. Previous serum protein data obtained from 98 healthy middle and upper income adults in Guatemala City were used for comparison.

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METHODS

Venous blood samples were collected during both morning and afternoon from ambulatory individuals and allowed to stand for 30 minutes before centrifugation. In the Guatemalan subjects the majority were mid-morning specimens. Total proteins in the sera were determined immediately afterwards by the gradient method of Lowry and Hunter.[9] Hemoglobin concentration was estimated in a sample of the fresh blood with a standardized Sahli hemoglobinometer. The remainder of the serum from each sample was packed in ice for air shipment to the central laboratories in Guatemala where analysis of the serum protein fractions was carried out by free flow electrophoresis in a Kern microelectrophoresis apparatus. A barbital buffer at pH 8.6 with an ionic strength of 0.1 was employed, and a current intensity of 200 milliwatts was applied for 35–45 minutes. Since with this method the separation of the alpha$_1$ globulin from the albumin is imperfect, these two fractions could not be estimated individually.

RESULTS

In Figure 1, the quartile values and limits of the total serum protein in 98 Panamanian subjects are compared with the control group of 98 adults from Guatemala City. The data show the relatively large number of high serum protein values among the Panamanians. Table 1 shows the average and range of total serum protein values for each of the three Panamanian groups selected for electrophoretic study together with the relative concentration of the protein fractions per 100 ml of serum. The average hemoglobin values for 79 of the Panamanian subjects was 13.8 ± 0.18. No relation between the serum

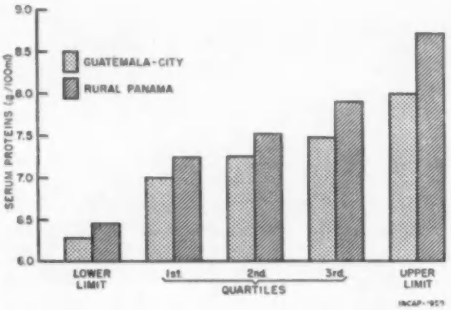

FIG. 1. Total serum proteins (grams per 100 ml) in adult subjects from "La Arena," Panama, and Guatemala City (98 individuals per group).

protein levels and the hemoglobin values was evident.

Analysis of the data suggests that the variation in protein within Group I (protein values below the median) was due mainly to differences in gamma globulin ($r = 0.846$, 7 *d.f.*). Although there were also small positive correlations with the albumin-alpha$_1$ and beta globulin fractions, these were not statistically significant. In contrast, the variation in total serum protein within Group II (values between second and third quartiles) seemed to be associated with differences in the albumin-alpha$_1$ globulin fraction, paralleled by a reciprocal change of approximately the same magnitude in the alpha$_2$ globulin. Nevertheless, both of these ($r = 0.655$ and -0.633 respectively, 7 *d.f.*) fell short of the 5% level of significance. Variations in the beta globulin seemed to have a very small effect on total protein, while those of the gamma fraction had none. Although the study was designed because of special interest in the behavior of serum protein fractions within Group III made up of individuals with serum protein values above 8.21 g per 100 ml, none of the correlations between the serum protein fractions and total serum protein reached statistical significance. The correlations (7 *d.f.*) were, however, positive for the gamma globulin fraction ($r = 0.633$), essentially zero for the alpha$_2$ globulin ($r = 0.010$) and negative for the beta ($r = -0.517$) and the albumin-alpha$_1$ globulin fractions ($r = -0.229$). Because of the limitations imposed by the relatively small size of the groups, the relationships suggested by the within-group analysis were examined by analysis of the pooled data. The analysis of the combined data for all groups indicated that while the groups differed in absolute concentration of the various fractions, the latter increased proportionally with total protein content and remained unchanged in their relative concentrations.

DISCUSSION

It has been previously pointed out that relatively high serum proteins may be found in population groups with relatively poor quality or quantity of dietary protein.[11] Although the most striking examples come from tropical regions, the phenomenon is also seen to a lesser extent in temperate zones, as illustrated by reports from Rochester,[11] Detroit,[10] and the Netherlands.[1] If it is due in any way to an inadequate intake of protein, it is not surprising that most reports come from the technically underdeveloped areas of the tropics where this type

TABLE 1

Electrophoretic fractions of serum proteins in adult subjects of La Arena, Panama (9 individuals per group)

Group	Serum total protein (g/100 ml)		Protein fractions in per cent of total proteins							
			Albumin + alpha$_1$ globulin		Globulins					
					Alpha$_2$		Beta		Gamma	
	$\bar{x}$	Range	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
I	7.24	6.54–7.39	59.0	3.51	9.6	1.93	13.7	2.16	17.7	1.98
II	7.72	7.63–7.90	61.6	2.15	7.7	2.52	14.6	3.06	16.1	2.61
III	8.34	8.21–8.55	60.4	4.18	8.5	2.33	14.4	3.06	16.7	5.99

of deficiency is exceedingly common and severe. Similarly, tropical areas suffer most from acute and chronic infections which may also be a factor in hyperproteinemia.

It has been recognized for some time that serum protein in the adults of at least certain populations in rural Panama were markedly higher than normal for well-nourished populations, despite their relatively poor diet.[12] This is strikingly shown by the comparison between the Panamanian subjects and the "control" Guatemalan group in the present study. The time of day at which samples are collected seems unimportant since Fawcett and Wynn[5] have shown no definite diurnal variation of serum protein levels except for the elevation of protein concentration on rising from bed. The same tendency as in the rural Panamanian adults was observed in a less striking fashion in rural school populations studied in several of the Central American countries.[11] It was apparent from the separation of protein fractions in a sample of the school children that the elevations could not be fully accounted for by increased globulins but must also be associated with a rise in albumin. The present study establishes that this is also true for the much higher serum protein values of rural Panamanian adults. The electrophoretic pattern of the hyperproteinemic sera was similar to that of a group of clinically healthy subjects from Guatemala City with total serum protein within the range accepted as normal.[4]

The fact that the Panamanian subjects were ambulatory clinically healthy individuals, consuming food and readily available water *ad libitum*, as well as the lack of correlation between the levels of serum proteins and the hemoglobin concentrations, suggest that dehydration is not playing a significant role in this phenomenon. At least three different mechanisms appear, however, to be operating to produce high serum protein values. In some studies[2, 3, 7, 15] they are explained by an elevation in the gamma-globulin fraction, while Holmes et al.[6] attributed the increase in total serum protein to a rise in the beta globulin fraction, and in the present study a proportionate increase in all fractions was observed. These differences emphasize the need to investigate the nature of the hyperproteinemias occurring in each specific population. Certainly a single general explanation does not seem to be applicable and the tendency to attribute all such increases to a specific increase in gamma globulin due to the prevalence of infection is unwarranted.

SUMMARY

A high prevalence of hyperproteinemias has been found among the inhabitants of La Arena in Panama in a random sample of 98 individuals. The values for the first, second and third quartiles were 7.24, 7.52 and 7.90 g of total protein per 100 ml of serum, with a range of 6.45 to 8.72. For electrophoretic studies, three subsamples of 9 subjects each were selected, with total serum protein values as follows: Group I, below the median; Group II, between the median and second quartile; and Group III, above the third quartile. Each of the electrophoretic fractions determined (albumin plus alpha$_1$ globulin, alpha$_2$, beta and gamma globulin) was proportionately responsible for the total serum protein elevation.

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ECOLOGICAL STUDIES OF WILD RODENT PLAGUE IN THE SAN FRANCISCO BAY AREA OF CALIFORNIA

III. The Natural Infection Rates with *Pasteurella pestis* in Five Flea Species During an Epizootic

S. F. QUAN, V. I. MILES, and L. KARTMAN*

In ecological studies of wild rodent plague, a matter of fundamental interest is the rate of natural infection with *Pasteurella pestis* in fleas especially during epizootic outbreaks. Such a rate of infection, based upon examination of individual fleas, has never been determined as it has in studies with anopheline vectors of malaria, for instance. In the past, the inoculation of pooled fleas into laboratory animals has been used to delineate the geographic extent of sylvatic plague[6] or to determine the presence of plague foci in rural areas where the infection may present a hazard to man[16, 17]. On the other hand, critical studies of wild rodent plague must now include determinations of natural infection rates if the epizootiology of the disease is to be more clearly assessed. The data reported here are concerned with *P. pestis* infection rates in fleas found during an epizootic of wild rodent plague in northern San Mateo County, California.

MATERIALS AND METHODS

The area studied and the methods used in trapping mammals and collecting their fleas have been described in the introduction to this series[13]. The details used in identifying *P. pestis* in fleas have been given in another previous paper of this series[18]. The essential steps are given below.

After the fleas were identified, they were placed one to a test tube (13 x 100 mm). Each flea was crushed and triturated with a sterile 3 mm glass rod which was moistened by dipping it into a shallow aqueous suspension of 1% "Attasorb". When the flea was well triturated, 0.2 ml of 1% peptone water was added and a suspension was made. The suspension adhering to the glass rod was streaked directly onto a section of a blood agar plate. In the early tests the residual suspensions of the triturated fleas were pooled and inoculated into mice immediately after the agar plates were incubated. Later, when it was found that a presumptive result could be made from the cultivation of the triturates after only 24 hours of incubation of the agar plates, pooling for animal inoculation was delayed. This delay allowed for separate inoculation of presumptive positive fleas and those that were highly contaminated, and yielded more information on individual fleas. The blood agar plates were incubated at 28°C, the optimum temperature for the growth of *P. pestis*[19], and were observed daily for at least 5 days.

Bacterial colonies of *P. pestis* were examined under the stereoscope for gross colonial morphology. Smears were made from them, and were tested for Gram and bipolar staining. Selected colonies were subcultured; *P. pestis* was confirmed by use of specific bacteriophage[3] and by animal inoculation.

The suitability and the efficiency of the individual cultivation procedure as compared to animal inoculation for the identification of *P. pestis* in fleas has been discussed in another paper[18]. It is sufficient to mention here that the in vitro procedure had a dependability equal to that of the in vivo method.

In this study, of 16 collections of fleas cultured, all but 3 collections were also inoculated into mice as a check against the cultivation technique. The suspensions of single or pooled flea triturates were inoculated subcutaneously into the right inguinal region of white laboratory mice (Princeton strain). Mice were observed for 10–14 days. All mice that died after inoculation were autopsied. Survivors were sacrificed and examined for pathology. The presence of *P. pestis* was confirmed in each case by culture, and when necessary by reinoculation into mice and guinea pigs.

RESULTS AND DISCUSSION

The data presented and discussed below are findings obtained during a 6-week period in a sylvatic plague epizootic which apparently began prior to the first week shown here. A total of 2,202 fleas, of 9 species, was collected and examined during this intensive investigation. *Malaraeus telchinum* was the most predominant flea in the area, consisting of 1,557 or 70.7% of

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TABLE 1

Natural infection rates of various flea species during a sylvatic plague epizootic among field mice in Northern San Mateo County, California; March 23 to April 30, 1954

Collection Week No.	*Pasteurella pestis* infection rates of indicated flea species																				
	Malaraeus telchinum			*Hystrichopsylla linsdalei*			*Catallagia wymani*			*Atyphloceras multidentatus*			*Opisodasys keeni nesiotus*			Other*			All species		
	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.
1	316	33	10.1	42	10	23.8	26	1	3.8	26	0	0	12	3	25.0	3	0	0	425	47	11.1
2	421	24	5.7	61	6	9.8	45	0	0	30	1	3.3	1	0	0	0	0	—	558	31	5.6
3	257	11	4.3	41	6	14.6	25	0	0	21	0	0	9	0	0	0	0	—	353	17	4.8
4	180	8	4.4	28	2	7.1	30	0	0	17	1	5.9	1	0	0	0	0	—	256	11	4.3
5	132	0	0	28	3	10.7	19	0	0	16	0	0	10	0	0	1	0	0	206	3	1.5
6	251	0	0	47	1	2.1	53	0	0	36	1	2.8	13	0	0	4	0	0	404	2	0.5
Totals	1557	76	4.8	247	28	11.3	198	1	0.5	146	3	2.1	46	3	6.5	8	0	0	2202	111	5.0

* All *Nosopsyllus fasciatus* except 1 *Corrodopsylla c. obtusata* in week 5; 1 *Monopsyllus w. ophidius* and 1 *Cediopsylla i. inaequalis* in week 6.

the total collected. Of the remaining fleas, 247 or 11.2% were *Hystrichopsylla linsdalei*; 198 or 9.0% were *Catallagia wymani*; 146 or 6.6% were *Atyphloceras multidentatus*; 46 or 2.1% were *Opisodasys keeni nesiotus*; and 8 or less than 0.4% consisted of 4 other species. Each weekly collection presented about the same composition of flea species; 60–75% *M. telchinum*, 10–14% *H. linsdalei*, 15–20% *C. wymani* and *A. multidentatus* combined, and about 5% *O. k. nesiotus* and other species.

Table 1 presents data on the infection rates of the various flea species for each week of the epizootic period under observation. Of the 2,202 fleas examined, 111 or 5% were found infected with the plague organism. The overall percentages of infection of the different flea species in descending order were as follows: *H. linsdalei*, 11.3; *O. k. nesiotus*, 6.5; *M. telchinum*, 4.8; *A. multidentatus*, 2.0; *C. wymani*, 0.5; and all others were negative.

Relation of Sex to Flea Infection Rates. Before presenting more detailed data on each flea species, it is of interest to examine the rates of infection in sexes of the 2 predominant flea species encountered during the plague epizootic. Only in the case of these 2 species of fleas, *M. telchinum* and *H. linsdalei*, the number collected and the number found infected were sufficiently large to analyze whether sex influenced the infection rates; these data are shown in Table 2. Of all the *M. telchinum* collected in the first 4 weeks, when the plague bacilli were present in this species, 65% were females, and 68% of the total found harboring *P. pestis* were females. With *H. linsdalei* similar percentages were obtained for the first 4 weeks, and for the 6 weeks the percentages of females collected and found infected remained about the same, 66 and 73 respectively.

The mean ratio of females to males for *M. telchinum* collected at 4 weeks and at 6 weeks was 1.9:1, while the ratio for those harboring *P. pestis* was 2.1:1. The ratio for *H. linsdalei* at 4 weeks was 2.4:1 and at 6 weeks was 1.9:1. For the infected fleas of this latter species the ratio at both times was 2.7:1. The *t*-test for significance was applied to the means of the sex ratios, with Yates's correction used for the small number of *H. linsdalei*. It proved positive. The 4-week mean was used in this test since no positive *M. telchinum* were found during the last 2 weeks. The results with both species showed no significant differences at 5% probability between the mean sex ratios of fleas collected as compared with the ratios of those which harbored *P. pestis*. In spite of the similarity of infection rates, it should be noted that females were found in greatest numbers in all flea species collected. Thus the female population may in itself constitute a factor in plague transmission. The importance of seasonal fluctuations in sexes of fleas has been noted by Cole[4].

Hirst[9] reported that plague transmission with rat fleas, *Xenopsylla cheopis* and *X. astia*, was

TABLE 2

Natural infection rates of Malareaeus telchinum *and* Hystrichopsylla linsdalei *by sex of the flea during a plague epizootic among field mice in Northern San Mateo County, California, March 23 to April 30, 1954*

Collection Week No.	*M. telchinum**						*H. linsdalei**					
	Male			Female			Male			Female		
	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.	No.	No. pos.	% pos.
1	120	13	10.8	196	18	9.2	15	2	13.3	27	7	25.9
2	141	7	5.0	280	17	6.1	16	1	6.2	45	4	8.9
3	90	2	2.2	167	9	5.4	16	3	18.7	25	3	12.0
4	56	2	3.6	124	6	4.8	9	0	0	19	2	10.5
5	52	0	0	80	0	0	8	1	12.5	20	2	10.0
6	83	0	0	168	0	0	20	0	0	27	1	3.7
Totals	542	24	4.4	1,015	50	4.9	84	7	8.3	163	19	11.6

* One positive *M. telchinum* and 2 positive *H. linsdalei* of unknown sex are not included

predominantly by females. On the other hand, Goyle[7] suggested that the males of the above 2 species were better hosts for the infection than the females. Since these early studies, a number of papers have been published in which conflicting evidence is reported in regard to the comparative efficiency of male and female fleas of various species as vectors of plague. Pollitzer[17] has summarized the literature in this regard. Although the weight of evidence seems to favor the hypothesis that the female flea is a more favorable host to *P. pestis* and a more efficient vector than the male, it must be pointed out that most studies have been based on small numbers of fleas, and the number of males used was often much smaller than the female sample. Recent work at this laboratory[12, 14] has shown that male *H. linsdalei*, with a higher infection potential than the females, gave a negative vector index. In these same studies, *X. cheopis* males had a higher vector index than the females on one occasion and a lower index than the females in another instance.

Although the data in Table 2 show no significant differences in the natural infection rates of male and female fleas, laboratory studies of the type cited above have made it clear that actual plague transmission is dependent upon factors affecting the flea after an infection has been established in its intestinal tract. Thus the speed of proventricular blocking (incubation period) and the length of survival of the blocked individual are critical factors in plague transmission[12]. It remains to be determined whether the infection potential, the blocking potential, and the survival of blocked fleas under natural conditions are comparable to these same indices obtained under experimental conditions. Such information will help to clarify the nature of vector efficiency as it actually occurs in sylvatic epizootics of plague.

Species of Fleas Infected with P. pestis. The 5 flea species shown in Table 1 are reported here for the first time as infected with *P. pestis* in nature. On the other hand, each of these species has been infected in the laboratory and, with the exception of *C. wymani*, each has, in experimental vector studies, transmitted plague to laboratory animals[1, 6, 21, 11, 14]. The important problem is the integration of laboratory observations with field data in evaluating the role of a given flea species in epizootics of sylvatic plague. Suggestions for such a correlation have been proposed by Kartman[10]. In the following discussion each flea species will be dealt with separately in more detail.

Malaraeus telchinum. Table 1 shows that *M. telchinum* was the most prevalent flea encountered on field mice during the 6 weeks of epizootic plague. Thus during the first 4 weeks, when *P. pestis* was present in this species, the total infection rates in all species of fleas combined closely resembled the rates in this species alone. The prevalence of this flea species and its presence on hosts throughout all seasons have stim-

ulated laboratory and other observations on its ability to act as a plague vector. Because of evidence that *M. telchinum* has been collected both on wild rodents and commensal rats[5], Stewart[20] postulated that this species might be an important vector of plague and that it might be capable of transferring the infection from sylvatic to domestic sources. Burroughs[1, 2] showed that this species could transmit plague in the laboratory primarily by infective feedings with groups of 25 to 100 individuals. Work at this laboratory (unpublished) has confirmed Burroughs' results. It seems clear that the mechanism of mass transmission may be of importance in plague ecology and, in the case of *M. telchinum*, this factor remains to be clarified.

The large population of this flea must be evaluated in relation to its known capability as a mass transmitter and its infection rate during the epizootic; *M. telchinum* made up 68% of all fleas found infected. During the first 4 weeks of the epizootic the infection rate varied from 10 to 4% (Table1). If fleas from only those hosts harboring infected fleas are considered during the first week, then the infection rate was 14% as compared with 10%. On some individual rodent hosts (*Microtus californicus*), the *M. telchinum* collected had infection rates as high as from 23 to 30%. In the laboratory, the following percentage infection rates have been obtained: 50 (Eskey and Haas[5]); and 27 (Burroughs[2]). The evidence seems fairly clear that, from the standpoint of prevalence, vector capacity, and infection rate, *M. telchinum* was an important vector of plague during the epizootic reported here. This suggestion is supported by studies with radioactively tagged *M. telchinum* which showed that this flea not only transferred between individual *M. californicus* but also from the wild rodent to *Rattus norvegicus* under simulated field conditions[8]. The finding of a plague-infected *R. norvegicus* during the epizootic[13] further suggests that *M. telchinum* may have been the vector by which the infection actually was transferred from a wild rodent to a commensal rat under conditions where populations of these hosts commingled.

Hystrichopsylla linsdalei. The population of this species found during the epizootic period was second only to *M. telchinum*, and its infection with *P. pestis* not only continued throughout the 6 week period but was consistently the highest of all species of fleas collected in the area (Table 1); *H. linsdalei* made up 25% of all fleas found infected. This species of flea was found most often on *M. californicus* with smaller numbers on other hosts. Furthermore, it has been shown in this area that *H. linsdalei* in *Microtus* nests had a *P. pestis* infection rate of 33% compared with a rate of 4% for *M. telchinum*[15].

In experimental studies, *H. linsdalei* has been shown to be an efficient vector by the mechanism of blocked individuals feeding on mice[11, 14]. Thus all evidence gathered to date on this species indicates that it was probably the major plague vector during the epizootic.

The consistent infection of *H. linsdalei* with *P. pestis* throughout the epizootic period studied suggests that this species could possibly be used as an indicator during plague epizootics in the San Francisco Bay area. The data show that, on rodent hosts with *P. pestis* positive fleas, the infection rate of *H. linsdalei* ranged from 33 to 67% with a mean of 50%, whereas *M. telchinum* varied from zero to 19% with a mean of 14%. Thus it may be possible to follow the course of a plague epizootic, in the specific ecologic area under consideration, solely by testing the *H. linsdalei*. In this way about 90% of the fleas collected would need no testing. In actual application, it was noted that the method could be used only during peak periods of epizootics when about 5% or more of the total fleas were proved infected with *P. pestis*.

Opisodasys keeni nesiotus. The extremely low population of *O. k. nesiotus* found on rodent hosts during the 6 weeks of the epizootic is shown in Table 1. Another matter of significance was the fact that infected fleas of this species were found only during the 1st week in which observations were made. It may be of interest to note that the 3 infected fleas found at that time were each taken off separate rodents of the three prevalent species, *M. californicus*, *Peromyscus maniculatus*, and *Reithrodontomys megalotis*.

In experimental studies Burroughs[2] showed that *O. k. nesiotus* had a 54% infection rate with *P. pestis* and was a fairly efficient vector to mice by the method of infective feeding of blocked individuals. This study by Burroughs also indicated a very short incubation period in this species of flea and the transmission ratios of *O. k. nesiotus* compared with *M. telchinum* were 0.06 and 0.04 respectively.

Studies on host relations show that *O. k. nesiotus* has a strong specificity for *Peromyscus maniculatus* in the area studied[15]. Where *Microtus* and

Peromyscus commingle it is possible that this flea could act in the transfer of plague from its preferred host to other hosts. In view of the meager population of *O. k. nesiotus*, its negative infection rate during 5 of the 6 weeks under observation, its ability to transmit plague, and its host specificity, it is suggested that this flea may have had a minor role in the epizootic among wild rodents.

Atyphloceras multidentatus. This species ranked fourth in the number of individuals collected from rodents during the epizootic. During this period only 3 of these fleas were found infected with *P. pestis*, as in the case of *O. k. nesiotus*. However, unlike the latter species, the infected *A. multidentatus* were found during weeks 2, 4, and 6 (Table 1). Over 90% of this species collected were females, and all 3 of the infected individuals were females. Studies in the area have shown that 15% of *A. multidentatus* found in rodent nests were infected with *P. pestis* and that this species occurs both on wild rodents and commensal rats[15].

Very little is known in regard to the plague vector efficiency of *A. multidentatus*. The only experimental evidence of its ability to transmit plague was obtained by Prince (unpublished, see Wayson[21]). Although Eskey and Haas[6] infected 20%, and Kartman and Prince[11] obtained an infection potential of 21% with this species, none of the individuals in these studies successfully transmitted the infection to laboratory animals.

On the basis of epizootiologic evidence, the abundance, natural infection, and host range of *A. multidentatus* during the plague outbreak, it is suggested that this species may have acted as a vector. However, until additional quantitative evidence in regard to its vector efficiency is obtained, no conclusions regarding its role during an epizootic can be made.

Catallagia wymani. The data in Table 1 show that this species was third in regard to total fleas collected, but ranked last in number of infected individuals found. Only one flea, a female, was found infected with *P. pestis* during the first week of the period under observation. This species had one of the widest host ranges of all fleas found in the area and thus could be of importance in transfer between various rodents. Practically nothing is known of its vector capacity except for the results reported by Eskey and Haas[6] who obtained an infection rate of 34% in the laboratory but no plague transmissions to experimental animals. Individuals of this species collected from a series of nests in the area were all negative for *P. pestis* infection[15]. Thus *C. wymani* appears to have been the least important flea species during the plague epizootic on the basis of currently available information. Obviously, a great deal of additional information is needed before a realistic evaluation of this species is possible.

Other Flea Species. Of the 2,202 fleas collected and tested during the 6 week epizootic period, only 8 belonged to species other than the 5 species shown in Table 1. Five of these were *Nosopsyllus fasciatus* and the remaining 3 were one each of *Corrodopsylla curvata obtusata*, *Monopsyllus wagneri ophidius*, and *Cediopsylla inaequalis interrupta*. None of these fleas was infected with *P. pestis*, and none was found on a host harboring other infected fleas. The finding of *N. fasciatus* reflects the commingling of the wild rodents with commensal rats and this flea could have served as a vector among the rats in the area. Although one rat was actually found infected with plague, the possibility of a rat epizootic was precluded by rat control operations in the area[15]. With the exception of *N. fasciatus*, the other 3 species of fleas mentioned above appeared not to have had any potential or actual connection with the sylvatic plague epizootic.

SUMMARY

From March 23 to April 30, 1954, a total of 2,202 fleas, composed of 9 species, was collected during a sylvatic plague epizootic among field mice in Northern San Mateo County, California. Of the total number of fleas obtained, the following are the species with their percentage occurrence: *Malaraeus telchinum*, 70.7; *Hystrichopsylla linsdalei*, 11.2; *Catallagia wymani*, 9; *Atyphloceras multidentatus*, 6.6; *Opisodasys keeni nesiotus*, 2.1; and 4 other species of minor importance, less than 0.4.

The rates of infection with *Pasteurella pestis* were determined by bacteriologic cultivation of individuals of all the flea species found. Of the total fleas collected, 111 or 5% harbored plague organisms. *H. linsdalei* had the highest and the most consistent infection rate throughout the period, with *M. telchinum*, *A. multidentatus*, *O. k. nesiotus*, and *C. wymani* following in descending order. Other species of fleas were negative for *P. pestis* infection. Of the total number of fleas found infected, *M. telchinum* constituted 68%, *H. linsdalei* made up 25%, and the other 3 species

together made up about 7%. All 5 of the above species were found naturally infected with *P. pestis* for the first time. The 2 main flea vectors of plague during the epizootic appeared to be *H. linsdalei* and *M. telchinum*.

Of the 5 major flea species examined, the predominant number of individuals were females. Separate data on infection rates in each of the sexes revealed little difference. A statistical test comparing the mean ratios of sexes of infected and uninfected *M. telchinum* and *H. linsdalei* showed no significant differences. Thus the sex of fleas seemed to have no relation to their natural infection rates with *P. pestis*. On the other hand, the predominance of females in the flea populations sampled suggests that, in this instance, the males may have played a more subordinate role in actual plague transmission to the rodents than the females.

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ECOLOGICAL STUDIES OF WILD RODENT PLAGUE IN THE SAN FRANCISCO BAY AREA OF CALIFORNIA

IV. The Fluctuation and Intensity of Natural Infection with *Pasteurella pestis* in Fleas During an Epizootic

S. F. QUAN, L. KARTMAN, F. M. PRINCE, and V. I. MILES*

In the present report, data are given to supplement the evidence discussed in Part III of this series[13] which indicated the *Pasteurella pestis* infection rates in several species of fleas during an epizootic of sylvatic plague in field mice which was studied from March 23 to April 30, 1954. Although the flea infection rate during an epizootic may suggest which species are the important vectors, the intensity of the infection and its fluctuation during the outbreak are of prime ecologic interest since they help to clarify some of the more obscure phenomena underlying the dynamics of wild rodent plague. Furthermore, these types of data from nature, although not always amenable to precise quantification, provide the fundamental background in the evaluation of experimental results.

MATERIALS AND METHODS

Details in regard to the area studied and much of the bacteriological methods have been presented in previous papers[6, 12, 13]. Usually, the fleas collected from host animals were placed in vials containing an aqueous solution of 2% NaCl and were then brought from the field to the laboratory for testing; each flea was triturated and cultured individually and/or inoculated into laboratory animals[13]. On April 7, however, fleas were placed individually into 13 x 165 mm test tubes in the field and were brought to the laboratory alive. These fleas were kept at room temperature overnight to allow them an opportunity to deposit fecal droppings in the tubes. On the next day, the fleas were killed, identified, and tested for infection as were other fleas. The test tubes were carefully examined for feces which were then tested bacteriologically for the presence of *P. pestis*.

Since each flea was observed individually under a microscope at the time of its identification, an opportunity was presented for the visual detection of *P. pestis*-like masses in the intestinal tract. When these were seen, the masses were dissected out and tested for plague organisms. Both intestinal masses and excrement were rubbed into suspensions by use of a sterile glass rod or a platinum wire loop on a glass slide or in a test tube. The suspensions consisted of a drop of flea material in 0.2 ml of broth which was then streaked onto a section of a blood agar plate and inoculated into mice in the same manner as the flea triturates.

RESULTS AND DISCUSSION

The Degree of Infection in Fleas. Of 111 fleas found positive for *P. pestis*, 90 or 81% were shown to be infected by bacterial culture, and 21 others or 19% were proved positive by animal inoculation only. The results are shown in Table 1. These data suggest a wide variation in the number of *P. pestis* in naturally infected fleas; the infection ranged from a few to millions of organisms per flea.

In Table 1 the last column shows the decline in the infection rates of all fleas during the epizootic as noted elsewhere[13]. However, the changes in degree of infection of individual fleas do not appear to follow this trend since substantial numbers of fleas yielding less than 20 colonies of *P. pestis* were found only during the first 3 weeks. Except for three fleas (one each from hosts in trap sites A-4, B-3, and D-7) taken during the 3rd week, all the individuals containing small numbers of plague bacilli were collected in trap sites yielding other fleas containing innumerable organisms.

The presence of small numbers of bacilli in a flea may have been due either to a recent incomplete feeding on a host with high bacteremia, a completed meal on a rodent with low bacteremia, or to the elimination of most bacilli from an in-

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TABLE 1

The intensity of Pasteurella pestis *infection in fleas during a sylvatic plague epizootic among field mice in Northern San Mateo County, California; March 23 to April 30, 1954*

Collection week No.	Number of fleas* with *P. pestis* shown by:				Totals	Per cent of all fleas positive‡
	Colonies on blood agar**			Animal inoculation†		
	Confluent	20–100	<20			
1	30	3	3	11	47	11.1
2	23	3	2	3	31	5.6
3	6	1	6	4	17	4.8
4	7	1	0	3	11	4.3
5	3	0	0	0	3	1.5
6	2	0	0	0	2	0.5
Totals	71	8	11	21	111	5.0
Per cent	64	7	10	19	100	

* Species of fleas were: *Malaraeus telchinum*, *Hystrichopsylla linsdalei*, *Atyphloceras multidentatus*, *Catallagia wymani*, and *Opisodasys keeni neseotus*.[13]

** The actual number of plague bacilli per flea was about 4 to 20 times the number of colonies on blood agar.

† In fleas proved positive by inoculation only; the degree of infection could not be evaluated.

‡ From Table 1 in Quan *et al.*[13]

fectious meal. The meal of low bacteremia appears to conform best with the data, although it has been a common observation that fleas are capable of "clearing" themselves of *P. pestis* at some stage after an infectious meal (see Pollitzer[9], for review). The phenomenon of clearing is at present but poorly understood, but it seems evident that it may be influenced both by environmental and other factors[6], and apparently it can operate at any intensity level of infection.

The presence of thousands to millions of bacilli in a flea was assumed to have been due to a recent feeding on a rodent in terminal septicemia or to the incubation of a culture established from an older infectious feeding. In this connection the predominance of confluent growth of cultures from fleas during the last 3 weeks of the epizootic is of interest (Table 1). If evidence for the decline of the epizootic during the last 3 weeks is assumed on the basis of a precipitous drop in the infection rates, then little or no new infectious blood meals could have been available to fleas during this period. Thus the preponderance of fleas with massive infections during the last 3 weeks suggests a reservoir mechanism which may be based on a slow development of *P. pestis* in certain species of wild rodent fleas under appropriate atmospheric influences. Even under more or less optimum conditions in the laboratory, the incubation period of *P. pestis* in various fleas may range from several weeks to several months[3, 5]. In the present instance, the fleas showing a preponderance of intense infections during the fourth week were *Malaraeus telchinum* and *Hystrichopsylla linsdalei*. On the other hand, only the latter species was infected and showed massive infections during the last two weeks of the epizootic. Experimental studies have shown that many individual *H. linsdalei* reached the stage of proventricular blockage with *P. pestis* after 30 days or more, although the mean incubation period was about 14 days[7]. These data again support the suggestion that *H. linsdalei* and *M. telchinum* were probably the two most important plague vectors during the epizootic[13].

Pasteurella pestis in Flea Feces and Intestinal Masses in Fleas. Of 148 fleas collected alive on April 7, 48 deposited fecal droppings in their test tubes overnight. Subsequently, 8 of the total fleas (5.6%) were proved positive for *P. pestis*, and 3 of the 48 fecal suspensions (6.3%) were shown to have viable plague bacilli. These results illustrate the difficulties involved in the testing of flea feces as an index of *P. pestis* infection. It is of interest that the results obtained from naturally infected fleas confirm experimental observations which have suggested that the culture of feces is a useful procedure but cannot provide an unequivocal basis for the determination of flea infection[5, 7].

Before the turn of the century, Simond[14] suggested that the entry of flea feces into bite wounds was the most important mechanism of plague transmission. Thus the Indian Plague Research Commission gave considerable attention to this mode of plague transmission by fleas[9]. Tumanski and Poliak[15] listed infection by rubbing of fecal droppings into skin wounds ahead of flea bites as a mode of plague transmission in rodents. However, experimental work has not confirmed these suggestions and flea feces have not been considered to be of importance in plague transmission under natural conditions[1, 3].

During the 2nd to the 6th week of the epizootic, 28 fleas were observed to have one or more dark masses in their proventriculi and/or ventriculi which were typically like those seen in laboratory infected fleas. On subsequent culture and animal inoculation, 9 of these fleas were proved to contain viable plague bacilli. These results confirmed experimental observations that visual criteria do not have a high correlation with final bacteriological proof[7]. Furthermore, it has been found experimentally that many individual fleas without visible masses in their intestinal tracts are infected with *P. pestis* when cultured[5]. Thus at the present time no conclusions can be drawn in regard to the appearance of "typical" plague masses in fleas from natural situations.

The Fluctuation of P. pestis Infection Rates in Fleas. The comparative ease with which *P. pestis* infections were detected in the wild rodent fleas collected during this study permits the presentation of data which indicate the length of time of the epizootic and the trend of the infection under natural conditions. In a previous report[13] the infection rates based on culture of individual fleas were presented in relation to 6 weeks of the epizootic starting on March 23, 1954. Nevertheless the epizootic actually began prior to that date as was indicated in the introductory paper[6]. Although no culture of individual fleas was done, *P. pestis*-positive flea pools were found in the area on March 10, 18, and 19. On the other hand, no positive fleas were found during earlier periods in October, 1953 and January, 1954. On February 25, however, a pool of 22 *H. linsdalei* collected from a nest of *Microtus californicus* was found positive for *P. pestis* infection. Thus it seems clear that the plague epizootic was in progress during February but that its peak occurred later when, fortunately, the culture of individual fleas had been initiated (see last column of Table 1). By the 6th week of this study (April 30, 1954) the infection rate in the fleas had declined to less than 1% when the epizootic was considered to have been terminated.

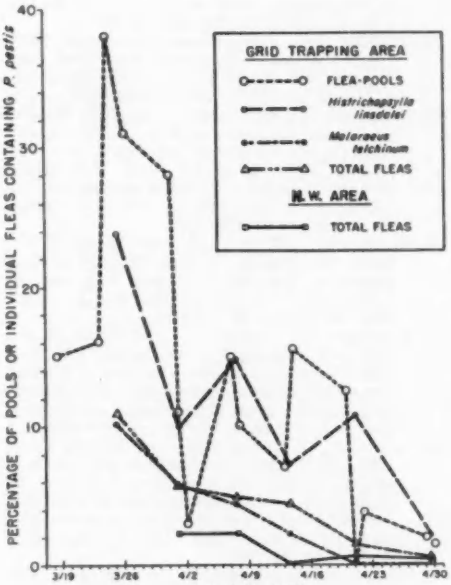

FIG. 1. The fluctuation of *Pasteurella pestis* infection in individual fleas and in flea pools during a sylvatic plague epizootic among field mice in Northern San Mateo County, California during 1954.

The fluctuation of the *P. pestis* infection in fleas during the epizootic is shown in Figure 1. This figure illustrates fluctuations in the two principal flea species *M. telchinum* and *H. linsdalei*, in all flea species combined, in all flea species collected in a contiguous plot northwest of the main trapping area, and in all positive fleas treated from the standpoint of pooled specimens rather than individuals. These data indicate that the peak of the epizootic, as shown by infected fleas, occurred during the week of March 26. In support of this suggestion, it should be noted that no rodents dead from plague were found on March 10 and 12, but that 15 dead *M. californicus* infected with plague were discovered during the week of March 26. Data presented in another paper showed that the area, in terms of square feet, from which infected fleas were collected was greatest during the first week and then steadily contracted during the next five weeks[8]. Quan and associates[10] have demonstrated that experimental plague epizootics have a fulminating character among susceptible hosts on which a large number of infected fleas were placed. In nature, it seems reasonable to assume that maximum spread of the disease would take from 2 to 3 weeks since it is dependent upon a build up of the population of infected fleas among hosts of varying susceptibility. In view of the data presented here, it appears that the wild rodent epizootic extended over a possible maximum of about 8 to 9 weeks and demonstrated vigorous activity for about 4 weeks. It has been observed that plague epizo-

otics in ground squirrels in California have a duration of from 2 to 3 weeks. On the other hand, Gerber[4] and Davis[2] have reported epizootics in African rodents to last for several months.

Some inferences may be drawn in regard to the factors responsible for the rise and fall of the epizootic wave during the period under observation. The dominant rodent in this area was the vole *M. californicus* which has been shown to be about 90% resistant to the intracutaneous inoculation of more than a million virulent plague bacilli (unpublished). The nature of this resistance has not been elucidated, but it has been shown that resistance to plague in *Microtus* varies according to area and whether the animals are wild caught or laboratory bred[11]. In the present studies there were also some indications that suckling young were very susceptible. Thus it appears probable that a limiting factor in this epizootic was the availability of rodents susceptible to plague infection. It is not clear whether both rodents and fleas acted as reservoirs of the infection, but once the epizootic started it has been shown that a large population of infected fleas was attained[13]. The presence of many infective fleas must have resulted in a rather rapid spread of plague among the small number of susceptible adult rodents and the majority of suckling young in their nests. Thus the rapid decline in the epizootic could have been due to the sudden depletion of susceptible animals. This explanation seems consistent with the picture shown here of the fluctuation of the infection in fleas during the epizootic period.

SUMMARY

Observations were made of the intensity of *Pasteurella pestis* infection in fleas collected during an epizootic of wild rodent plague. The number of plague bacilli in infected fleas had a wide range of from a few to millions. The degree of infection in the fleas during 6 weeks of the epizootic showed that whereas fleas with high and low numbers of bacilli were present during the first 3 weeks, those found infected during the last 3 weeks had massive infections exclusively. Thus in certain species, like *Malaraeus telchinum* and *Hystrichopsylla linsdalei*, intense infections during a period of epizootic decline may represent a reservoir mechanism.

There was a generally poor correlation between infection rates of individual fleas and their fecal droppings. Likewise, visual criteria in regard to the presence of "typical" plague masses in the intestinal tract of fleas were but poorly borne out by subsequent culture of the same individuals. These findings confirmed former experimental results.

Observations of the fluctuation of *P. pestis* infection in fleas suggested that the epizootic lasted for approximately 8 weeks with vigorous activity during the middle 4 weeks. The peak period occurred during the third week, March 26, 1954, and the infection rate declined to low levels in the next 3 weeks and finally terminated on about April 30. It appeared probable that a limiting factor in the epizootic was the availability of rodents susceptible to plague.

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ECOLOGICAL STUDIES OF WILD RODENT PLAGUE IN THE SAN FRANCISCO BAY AREA OF CALIFORNIA

V. The Distribution of Naturally Infected Fleas During an Epizootic in Relation to Their Infection Rates

L. KARTMAN, S. F. QUAN, AND V. I. MILES*

From the earliest empirical investigations on the epidemiology of bubonic plague to the present era of quantitative ecologic study, the problem of the spread of the infection in nature has intrigued research workers. Although the spread of rat-borne plague could, in most cases, be explained especially on the basis of human agencies such as the ships of international commerce, the problems relating to the spread of wild rodent plague have not been so amenable to elucidation. Even to-day it still appears to be a moot question whether plague has spread from the west coast port cities of the United States eastward to the 100th meridian, or whether the infection has been resident in "archeological" foci for a period antedating the arrival of man and his commensal rats.

Obviously, the spread of plague is directly affected by the movements and habits of its principal mammalian hosts and flea vectors. Thus its spread is intimately linked with the mode of its persistence and the ecologic nature of the areas in which it exists. Although workers have from time to time drawn attention to the necessity for an ecologic approach, it was Pavlovsky[7] who evolved what has become known as the "doctrine of nidality" (i.e., focal localization). This concept, which has been a fruitful one for Soviet scientists, emphasizes the precept that a disease, like a species, itself tends to have a natural habitat. Thus the zoonoses have habitats in clearly defined ecosystems where the pathogenic organisms, the vectors, and the disease-affected hosts form associations or biocenoses within which the pathogenic cycles operate. Audy[1] has extended and developed these ideas in relation to various tropical infections.

In the study of an outbreak of wild rodent plague in northern San Mateo County, California, an attempt has been made to present evidence relating to some of the ecologic factors involved (see introductory paper[3]). A related study was reported by Murray[6] who presented much of value on the plant-animal-flea associations found in areas of epizootic plague. In the present paper, evidence is presented on the distribution of infected fleas during a 6 week study of an epizootic in relation to the infection rates of these vectors. The changes occurring in the distribution and infection of the fleas within a well defined natural habitat is of fundamental interest in the elucidation of factors governing the epizootic.

MATERIALS AND METHODS

A description of the study area, the field collecting methods, the grid pattern of trap lines and trap sites established, and information relative to vegetative cover and other ecologic associations have been presented in the introductory paper[3]. Of special importance to the present report is Figure 2, in the introductory paper, which is a diagram of the study area showing the locations of individual trap sites and their environment. When trap lines and/or trap sites are mentioned here, they refer to locations shown in the above mentioned figure.

The bacteriological methods for determination of *Pasteurella pestis* infection rates in fleas, and for confirmation of the presence of plague organisms have been delineated elsewhere[8, 9, 10].

The work in an area contiguous with the main study plot was accomplished by the same methods. The trap lines and trap sites in the adjacent area, west and northwest of the main grid, are also shown in the figure mentioned above. Data from this adjacent area were obtained only during the second to sixth weeks of the investigation.

RESULTS AND DISCUSSION

It should be noted at the outset that previous work indicated that the region in which the epizootic study area was located has a history of plague. In 1942, three foci of plague were found on the basis of ectoparasites collected from ground

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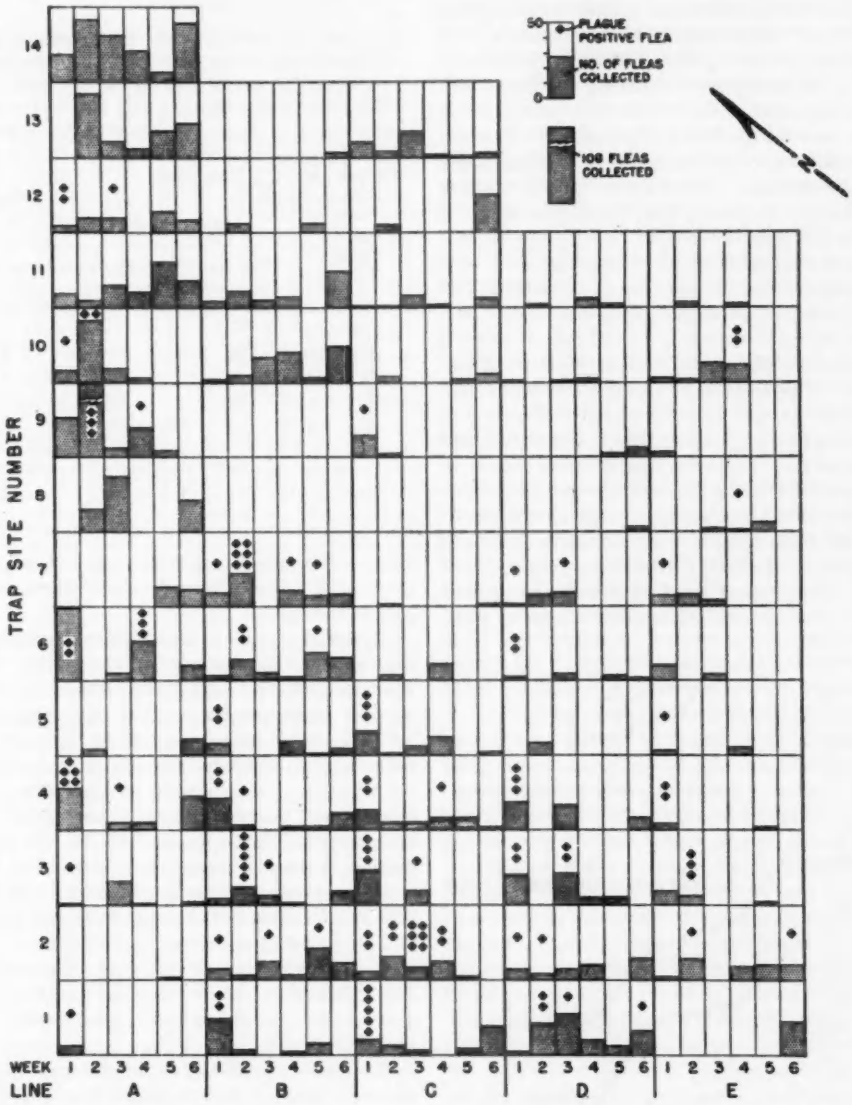

FIG. 1. The density and distribution of fleas infected with *Pasteurella pestis*, and trap sites yielding rodent hosts with infected fleas, during 6 weeks of a sylvatic plague epizootic in Northern San Mateo County, California; March 23 to April 30, 1954 (the plot measured about 200 x 600 feet; traps were set 50 feet apart in a grid of 5 trap lines).

squirrels and inoculated as pools into laboratory animals[6]. Furthermore, during March and April of 1954, five plague foci were found approximately 100 to 300 feet east, and from 100 to 3000 feet northwest of the study area. During March, 1955 a plague focus was found a little over a mile north of the study plot[3]. Thus, although plague had never been recovered from the actual study area

before, its discovery there in 1953[5] was consistent with the known history of the region. A map showing sylvatic plague findings in this region gives the impression of delimited foci from which the dispersion of the infection *per saltum* would not have a high degree of probability[3]. This impression was supported by plague findings during April, 1957 in one of the foci previously shown to be positive in March, 1955 (unpublished).

In the present study the main epizootic area was an approximately trapezoidal plot measuring 200 by 600 feet. In this plot, a grid pattern of 62 trap sites, at 50-foot intervals, was laid out in 5 trap lines. The general distribution of infected fleas in the plot during 6 weeks is shown in Figure 1. This figure is a diagrammatic representation of the epizootic area shown in Figure 2 of the introductory paper[3]. The concentration and distribution of infected fleas occurred mainly in that portion of the plot characterized by a loam-type soil and coastal brush vegetative cover in which dense tangles of coyote brush (*Baccharis pilularis*), blackberry (*Rubus vitafolius*), lizard tail (*Briophyllum staechadifolium*) and various herbs were found. The contiguous areas of cultivation on the south and southwest, and heather on the east and southeast, produced little or no plague findings.

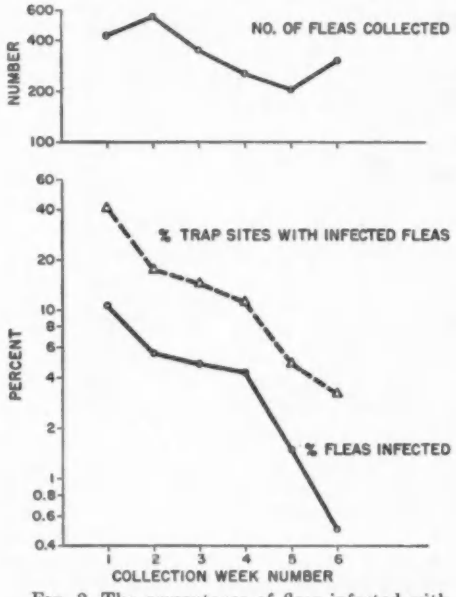

FIG. 2. The percentages of fleas infected with *Pasteurella pestis*, and trap sites yielding rodent hosts with infected fleas, during a sylvatic plague epizootic in Northern San Mateo County, California; March 23, to April 30, 1954.

TABLE 1

Comparison of the combined Pasteurella pestis *infection rates in the fleas* Malaraeus telchinum *and* Hystrichopsylla linsdalei *in the main epizootic focus and in a contiguous area to the west and northwest during a sylvatic plague outbreak in Northern San Mateo County, California; March 31 to April 30, 1954.*

Collection week No.	Combined infection rates in:					
	Main area			Northwest and west area		
	No.	No. pos.	% pos.	No.	No. pos.	% pos.
2	482	30	6.2	153	4	2.6
3	298	17	5.7	110	3	2.7
4	208	10	4.8	176	0	0
5	160	3	1.9	134	1	0.7
6	298	1	0.3	177	1	0.6
Totals	1446	61	4.2	750	9	1.2

The infection rates in the individual flea species have already been noted[9]. It was shown that *Malaraeus telchinum* and *Hystrichopsylla linsdalei* were the most abundant and most consistently infected species during the 6 weeks. It is of interest here to compare infection rates in these species with rates in the same species in the contiguous area to the west and northwest of the main epizootic focus. These data are shown in Table 1. Although 3 other species were found infected in the main epizootic area, only *M. telchinum* and *H. linsdalei* harbored *Pasteurella pestis* in the adjacent plot referred to in Table 1. It is also of interest that 50 rat fleas, *Nosopsyllus fasciatus*, were collected from 18 *Rattus norvegicus* in the latter plot, but all were negative for *P. pestis* infection.

The higher infection rates in the main area, shown in Table 1, suggest that the infection of fleas in the adjacent plot to the west and northwest may have been due to a spread of the disease by contiguity, a mechanism which has been ascribed to "an accumulation of small movements among rodents"[4]. The trap sites in the adjacent area ranged from 50 to about 400 feet from the epizootic focus. During April, 1954 a plague-infected rat, *R. norvegicus*, was found at trap site R3 which was about 150 feet from trap

TABLE 2

Changes in the extent of the area in which fleas, infected with Pasteurella pestis, *were found during an epizootic of plague among field mice in Northern San Mateo County, California; March 23, to April 30, 1954.*

Collection week. No.	No. trap sites with infected fleas in the following trap lines;					No. trap sites with infected fleas		Total area (sq. ft.) with infected fleas (no. trap sites × 2500*)
	A	B	C	D	E	Total	New	
1	6	5	6	5	3	25	—	62,500
2	2	4	1	2	2	11	6	27,500
3	2	2	2	3	0	9	0	22,500
4	2	1	2	0	2	7	2	17,500
5	0	3	0	0	0	3	0	7,500
6	0	0	0	1	1	2	0	5,000
Total trap sites	12	15	11	11	8	57	8	

* Each trap site was approximately 2,500 square feet.

site A1 in the epizootic area. In view of the negative findings in rat fleas, this adds further evidence to the suggestion that the infection spread through short movements of wild rodents by which infected wild rodent fleas were transferred to domestic rats and other wild rodents from the main epizootic area to the contiguous area.

During the 6 weeks of observation of the plague epizootic, 33 different trap sites were found to harbor hosts with *P. pestis*-infected fleas. During the same period, infected fleas were found in a grand total of 57 trap sites, some of which yielded positive fleas from 2 to 4 times (Figure 1). The fluctuation of trap sites with infected fleas closely followed the variation in flea infection rates. These data are shown in Figure 2. The steady decline of both positive trap sites and positive fleas suggests that whatever basic influences effected the fall and termination of the epizootic, any favorable microenvironmental factors which the area offered could not overcome those influences. Some reasons for the epizootic decline are suggested in another paper[10].

The data presented above suggest that although there was apparently a small spread of the infection from the epizootic area to a contiguous area, the amount of spread was severely circumscribed. Furthermore, there was no evidence of spread of infected fleas beyond the confines of the study area or of a connection with nearby plague foci. Some evidence which shows the spread of the infection within the epizootic area is presented in Table 2. New trap sites yielding hosts with infected fleas were found during the second and fourth weeks. An analysis of the data in Figure 1 suggests that the new positive trap sites tended to be those to the east of the majority of positive trap sites which were found during the first week and which continued to yield infected fleas during subsequent weeks. These observations suggest that small movements among rodents and the transfer of fleas from host to host may account for the small amount of plague spread that occurred during the epizootic. In this connection, it should be noted that the transfer of fleas between hosts of the same and different species has been demonstrated[2].

Further evidence of the pocket-like nature of the epizootic may be obtained by noting the amount of space encompassed by the infection in fleas during each of the 6 weeks. This type of analysis is presented in Table 2. These data clearly show a steady contraction of the affected area; no evidence of increase in the area was found after the first week except for a slight shift in the location of positive trap sites as indicated above. This trend was characterized by a decline in total trap sites per week and in the number of new trap sites yielding infected fleas during the same period.

The above discussion in regard to possibilities of plague spread makes it necessary to mention the commensal rats at greater length. It has been indicated above that a Norway rat was found dead from plague in a trap site contiguous with the first trap line of the main epizootic area. This contiguous area extended westward to a willow grove close to structures of the hog farm where the rats occurred. During a period of 5 weeks the following rodents were trapped in this area: 123 *Microtus californicus*, 14 *Peromyscus maniculatus*, 18 *Rattus norvegicus*, and 1 *Mus musculus*. The *P. pestis* infection rates in fleas found on these rodents (primarily *Microtus*) have already been given in Table 1. The commingling of these wild and domestic rodents, the presence of an active plague epizootic, and the presence of infected fleas which are known to be capable of transfer from wild rodents to rats[2] suggests that the stage was set for spread of the infection into the rat population with a possible extension of the

epizootic area into the hog farm itself. The finding of a plague infected rat is concrete evidence for this possibility. Theoretically, it appears that a secondary involvement of the rat population would have been the only way in which the infection could spread beyond the confines of its natural localization. That this did not happen was undoubtedly due to the prompt action taken by local health authorities to control the rats by poisoning.

SUMMARY

Observations were made for 6 weeks on the distribution of infected fleas during a plague epizootic among wild rodents. The area harboring the greatest concentration of infected fleas was about 120,000 square feet of coastal brush vegetative cover on a loam-type soil. Infected fleas were also found in a contiguous area to the west and northwest of the main epizootic focus. The much lower activity of the infection in this adjacent area suggested that it had been initiated by spread from the epizootic focus.

The evidence from the distribution of infected fleas and their infection rates suggests that the plague epizootic among the wild rodents was strongly localized. No instance of spread of the infection at a distance was noted. The extent of the area in which infected fleas were found contracted steadily from a maximum of over 62,000 square feet during the 1st week of observations to about 5,000 square feet in the 6th week. This shrinkage was accompanied by a decline in total trap sites and in the number of new trap sites yielding infected fleas each week, and by a concomitant decline in flea infection rates.

The presence of commensal rats in a plot contiguous to the epizootic focus, and the finding of a *Rattus norvegicus* dead from plague, suggested that the avenue of possible major spread was into the rat population. This eventuality appeared to have been precluded by a prompt rat control campaign by local health authorities.

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Preventive Medicine: Principles of Prevention in the Occurrence and Progression of Disease, edited by HERMAN E. HILLEBOE, M.D. and GRANVILLE, W. LARIMORE, M.D. xxxi + 731 pages. Philadelphia and London. W. B. Saunders Company, 1959, $12.00.

With the field of Preventive Medicine constantly commanding more of the interest and responsibility of the practicing physician, it is gratifying to welcome this splendid new textbook written primarily for medical students and practitioners. The book distinguishes between prevention of occurrence of disease, the primary goal, and prevention of progression, the secondary goal; and the main division of the text is along these lines. Of the 31 contributors, all but three are present or former members of the staff of the New York State Department of Health, and the text is prepared largely from material used in lectures to medical students, physicians and health department employees.

Under Prevention of Occurrence, the first eleven chapters deal with control of environmental factors including water, food, waste, air, insects, radiation, disasters, accidents, housing and occupation. Then follow six chapters on prophylactic measures against specific groups of infectious diseases, a chapter on nutrition and three chapters on the elimination of predisease conditions, namely obesity and the health hazards of childbirth and childhood. Two chapters on preventive dentistry conclude Part I of the book.

Part II, Prevention of Progression, opens with a chapter on periodic health inventories, followed by five chapters on early detection of the insidious diseases by screening for cancer, tuberculosis, heart disease, diabetes, hearing and visual defects, and a chapter on the follow-up of screening and diagnostic examinations. Chapters on rehabilitation, alcoholism and narcotic addiction complete Part II.

Part III deals with Supporting Services for Preventive Medicine, including education of the public and patients, and postgraduate education of physicians. Finally, a section on services aiding the practice of Preventive Medicine includes chapters on social work, public health nursing, the role of the hospital in preventive medicine, epidemiology, and official and voluntary health agencies.

This reviewer has been fascinated by the clarity, conciseness and "uptodateness" of this text. The uniformity of excellence among so many contributors is unusual. Continual application of the subject matter to the practicing physician, and omission of unnecessary details, hold the interest of the unspecialized reader. The 33 tables and 59 text figures add much to the clarity of presentation. The only chapters which give details not important to the practitioner are those on water purification and excreta disposal. The only noteworthy omission is a chapter on mental health, which is given only casual consideration under other headings.

The format of the book is very attractive and the editing has been almost perfect. This reviewer noticed no typographical errors and only two editorial ones. The volume is a notable contribution to the practice of preventive medicine, and should be used widely as a text for medical students and as a guide for physicians interested in adapting their practice to the future needs of patients and the community.

HENRY E. MELENEY

Microbiology—Yesterday and Today. A Symposium, VERNON BRYSON, Editor. 122 pages illustrated. New Brunswick, New Jersey, Institute of Microbiology, Rutgers, The State University, 1959. $4.00.

Reading a symposium is like enjoying a banquet via radio. Participation would be so much more satisfying! But the printed word in this book conveys a very good idea of the substance, if not of the savor, of the intellectual *pièce de résistance*. The Savor is, of course, the inspiration of personal contacts with not only the principal speakers but with the moderators, the organizers and the audience. The Substance is presented, as a tribute to the life and work (up to his 70th birthday, so far) of Dr. Selman A. Waksman, founder of the Institute of Microbiology at Rutgers, in the form of 7 lectures: 1. Microbial Biochemistry and Its Development, J. H. Quastel; 2. Antibiotics—A New Field for Microbiological Research and Perspectives for

the Future, H. Boyd Woodruff; 3. Episodes in Immunochemistry, Michael Heidelberger; 4. Bacterial Classification—Problems and Developments, S. T. Cowan; 5. Some Contributions of Genetics to Microbiology, Vernon Bryson; 6. Aspects of Russian Microbiology, George K. Skriabin; 7. Microbiology—Yesterday and Today, Selman A. Waksman. The lectures are not stilted scientific presentations but are summaries, outline anecdotal histories, viewpoints, ideas, cogitations based on scientific data. The standing of each speaker vouches for the quality of the mental nutrition and the reader easily digests very weighty scientific fare, seasoned as it is with metaphorical *bons mots* and epigrammatic plums. In short, the symposium is fascinating, informative, and stimulating; an ornament to any scientific library.

MARTIN FROBISHER

Malariology, with Special Reference to Malaya, by A. A. SANDOSHAM, L.M.S. (Singapore), PH.D. (London), F.R.E.S., F.L.S., F.Z.S., F.R.M.S. 1st edition, 327 pages, illustrated. Singapore, University of Malaya Press (New York: University of Oxford Press), 1959. $8.00 and £1.15s.

For those who may think that malaria is no longer a problem, this book may be a reminder that there are still parts of the world where it is still a disease of major importance. As the author remarks, "... the loss sustained by Malaya (from malaria) was colossal" in earlier days, and "*Kampong* or rural malaria has always remained the most difficult problem in the country." To help the medical student and the general practitioner to learn to cope with it, this book has been written. It is essentially a rather elementary manual of malariology, but it includes much that will be of general interest, even though intended to meet the needs of a Malayan audience. After a foreword by Dr. John W. Field, who is himself well acquainted with the problem of malaria in this part of the world, the author proceeds with a brief account of Malayan geography, climate, population, agriculture, and other factors relevant to the incidence of malaria and its control. Then there is a history of the disease in Malaya and of earlier, partially successful attempts to curb its ravages, but the major part of the book is concerned with the biology (in its broadest sense) of the parasites themselves, and of their anopheline vectors. The techniques of malaria control are, of course, well described. Among the most useful features of the book is the extended treatment given anopheline mosquitoes of Malaya, and the detailed directions for recognition of the various species of plasmodia. This is made easier by the inclusion of 11 colored plates of very high quality.

Though this is a text which can be highly recommended to those wishing only an introduction to malariology, there are some features conspicuously missing, such as any mention of the animal malarias or of the physiology of the parasites. Likewise, the pre-erythrocytic and exoerythrocytic cycle receives a scant paragraph. Nevertheless, the book is likely to meet very well the needs of those for whom it was written.

REGINALD D. MANWELL

A Synopsis of Skin Diseases, by BETHEL SOLOMONS, JR., M.A., M.D., F.R.C.P.I. 293 pages, illustrated. Baltimore, The Williams and Wilkins Company, 1959. $6.75.

The author has attempted to outline subject material useful for the busy practitioner, the house staff officer, and medical student. Most of the common dermatoses are covered. As with many synopses, readability and orderly classifications suffer. The absence of illustrations and lack of current therapy may detract from its value for the student. As a synopsis it lacks continuity albeit there is a wealth of material presented.

J. L. C.

Principles of Administration Applied to Nursing, by H. A. GODDARD (W.H.O. Monograph Series No. 41) 108 pages. Geneva, World Health Organization, 1958, £1, Sw.Fr. 12, $4.00.

This volume of just over 100 pages contains a wealth of material which should be helpful not only to those nurses who are in a position requiring the use of management knowledge and skills, but to those whose responsibility is to teach and help prepare others for assuming administrative responsibilities.

The purposes of the book are clearly stated in the preface and the material included is pertinent and clearly presented.

The overall organization of the material is logical and systematic. The principles basic to the science of administration are followed by a

discussion of the application of these principles specifically to health agencies. The greater part of the book is devoted to techniques of administration and methods for on-the-job training in the use of these techniques.

The portion of the publication devoted to "Annexes" is good and should prove extremely helpful, as the sample forms included are well set up and clear.

The Bibliography is extensive and carefully chosen and the annotations clear and concise.

Some of the outstanding strengths are the inclusion of the public health agencies, as well as hospitals in making application of principles and techniques; the emphasis throughout on administration as a process carried on by all persons at the various management levels; the stress on the importance of a systematic, careful planning for any activity based on fact-finding; the importance of informal as well as formal group discussion, and such concepts as participation in policy-formulation by those involved in carrying out the policy. The examples used, some simple, some more complex, are familiar and for the most part serve their purpose of clarification.

The scope of the book is broad, and depth in any of the areas included would therefore need to be supplemented. However, the material has been handled in a systematic, clear and concise manner which meets the stated purposes, i.e., a guide and a manual. As such it can be used profitably by those with some experience and sophistication in administration, both for help in functioning and in teaching others.

FRANCES K. CLYDE

BOOKS RECEIVED

Malariology, with Special Reference to Malaya, by A. A. Sandosham, L. M. S. (Singapore), Ph.D. (London), F.R.E.S., F.L.S., F.Z.S., F.R.M.S. 1st edition, 327 + xix pages, illustrated. Singapore, University of Malaya Press (New York: University of Oxford Press), 1959. $8.00, £1.15s.

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A Synopsis of Skin Diseases, by Beth Solomons, Jun. M.A., M.D., F.R.C.P.I. 1st edition, 293 pages, illustrated. Bristol, England John Wright & Sons, Ltd. (Williams and Wilkins Company, Baltimore, U. S. Agents), 1959. $6.95.

Coprologie Parasitaire Humaine (in French), by J. Bailenger, Fac. de Médecine et de Pharmacie de Bordeaux. 1st edition, 227 pages, illustrated. Bordeaux, E. Drouillard, 1959.

The Human Integument, by Stephen Rothman, editor, with 13 contributors. 1st edition, 260 + x pages, illustrated. Washington, D. C., American Association for the Advancement of Science, 1959. $5.00.

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which will entail a certain delay in their appearance because of the contractual limitation on the number of journal pages available in any year. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

1. *Manuscripts* should be typewritten in *English* using *double spacing* on *one side* only of 8½ x 11 inch paper and submitted *in duplicate*. Double-spacing should be used throughout the manuscript including the list of references, footnotes, and quotations.

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